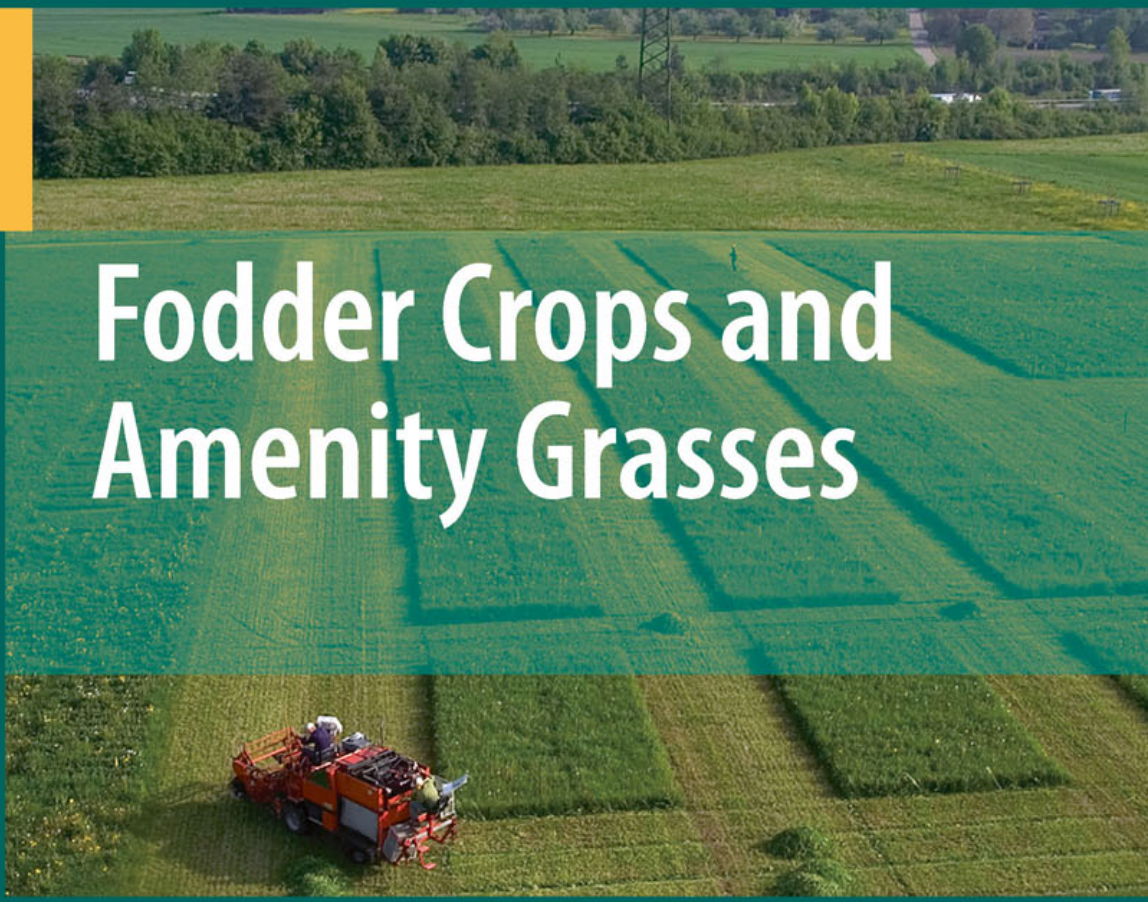
HANDBOOK OF PLANT BREEDING

Beat Boller
Ulrich K. Posselt
Fabio Veronesi
Editors



Fodder Crops and Amenity Grasses

Springer

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HANDBOOK OF PLANT BREEDING

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Preface

Grassland farming in Europe was already established during the settlement of the first farmers together with their domesticated animals after the last ice age. Since then, grassland provides the forage basis to feed ruminant animals for the production of meat and milk. Depending on the ecological conditions and intensity of usage, various plant communities with different species developed, displaying a rich biodiversity. With the introduction of improved crop rotations at the end of the 16th century, grasses and legumes were also grown to an important extent as forage crops on arable land. In the last decades the importance of amenity grasses increased markedly, due to the demand of the society for new usages like landscape protection.

Around 1900 interested farmers and academics identified the need for grassland improvement through systematic selection and seed production. This marks the beginning of breeding and research in companies but also at universities and specialized research institutes. Plant collection started with many of the species that are still of importance today. The collected materials were grouped according to the intended use and some type of phenotypic selection was applied. Seed multiplication of such populations was performed in pure stands and the harvested seed was marketed. Although the vegetative biomass and its quality are of utmost importance in forage crop breeding, it is the seed yield potential which determines the commercial success of a new variety.

There are some milestones in forage crop breeding that should be mentioned: the invention of the polycross leading to the replacement of open pollinated varieties by synthetic varieties, progeny testing, breeding of amenity grasses, induction of tetraploids in the ryegrasses and red clover, and the introduction and application of molecular tools. The invention of the forage plot harvester, computers, NIRS, and other new technologies has led to a tremendous increase in breeding intensity. Unfortunately, public funded research is decreasing dramatically in most highly developed countries, while in the commercial sector a concentration process took place. Thus, efforts are needed to avoid loss in knowledge and breeding experience.

Scientific and practical knowledge of forage plant breeding accumulated in the first 50 years of systematic fodder crop breeding has been summarized in the so far unique volume “Züchtung der Futterpflanzen – Breeding of Forage Plants” which appeared as the fourth volume of the bilingual “Handbuch der

Pflanzenzüchtung – Manual of Plant Breeding" in two editions, 1941 and 1959, and was edited by H. Kappert and W. Rudorf. In their foreword to the second edition, we can read that "the research results are scattered in profuse literature which can no longer be overlooked by the individual." Now, another 50 years later, this is certainly true even more and we as editors of the "Fodder Crops and Amenity Grasses" volume of this new "Handbook of Plant Breeding" are proud to tackle again the challenge of making the most pertinent knowledge available to the plant breeding community.

Because forage crops have many topics in common and to avoid redundancy, we decided to start with nine general chapters devoted to the role of forage crops in multifunctional agriculture, genetic resources, breeding methodology, molecular tools, breeding objectives in forages as well as amenity grasses, breeding for seed yield, variety testing and release, and an outlook into the future. The second part comprises the nine most important groups of temperate species among the grasses, clovers, and alfalfa. Minor species are also treated in respective chapters. Each of the crop-specific chapters covers the whole range of topics related to breeding from the origin and history of the particular crop and genetic resources to breeding achievements, specific goals and techniques, including the potential and actual integration of new biotechnologies. The chapters have been written by outstanding breeders and scientists with wide experience in their crops and topics.

This volume contains all the basic and updated information on the state of the art of breeding fodder crops and amenity grasses. The vast amount of knowledge collected in this volume should not only serve breeders as well as researchers, students, but also their academic teachers. It may be regarded as a scientific knowledge platform which provides practical plant breeders with new scientific information, but also to make molecular biologists more familiar with the peculiarities of breeding the various species of fodder crops and amenity grasses.

The completion of this book would not have been possible without the contributions of the many authors, who have devoted much time to the task of writing the chapters. The scientific platform of the Fodder Crops and Amenity Grasses Section of EUCARPIA has been an extremely valuable resource of recruiting highly competent contributors. We also want to thank the staff of Springer, in particular Hannah Schorr, for their continuous support.

Last but not least, we would like to thank Christine, Brigitte, and Daniela for their patience and support while working on this volume.

Zürich, Switzerland — Beat Boller
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The Role of Forage Crops in Multifunctional Agriculture

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1 Introduction

UNESCO defines grassland as "land covered with herbaceous plants with less than 10 percent tree and shrub cover". In many cases, grassland is grazing land.

According to the World Resource Institute, cited in Suttie et al. (2005), grasslands are among the largest ecosystems in the world. The area is estimated at 52.5×10^6 km^2, representing 40.5% of the terrestrial area, excluding Greenland and Antartica.

Since almost all European grasslands are more or less modified by human activities and have to a major extent been created and maintained by agricultural activities, they can be defined as "semi-natural" grasslands, although their plant communities are natural (Reidsma et al., 2006).

The multifunctionality of grassland as described in this chapter, considers grassland as a source, as a sink and as the combination of both source and sink.

2 Grassland as a Source

2.1 Grassland as an Indispensable Source of Nutrients: A Historical Perspective

Historically, grassland and some forage crops played a major role in the agricultural development in most parts of Europe. Semi-natural grassland has been for a very long time a "mine of nutrients" with the vegetation serving as miners and livestock as transportation belts.

Indeed until the industrial production of fertilizers, mankind faced the problem of nutrient depletion in agriculture. The more the population grew, dwelled into cities and the more industrial crops were grown, the fewer nutrients could be recycled. Since more people means an extra need for food, the major way to enhance

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food production was the expansion of the agricultural area for a very long time. The most fertile soils were dedicated to arable crops (mainly cereals), managed in a 3-year rotation system: 2 consecutive years of cereal production followed by a fallow period of 1 year.

Livestock grazed in the forest or on less fertile semi-natural grassland (managed as common land) and was brought home to rest on the fallow plots overnight, fertilizing the fallow land with their excrements. Hence livestock served as a "nutrient pump" pumping nutrients from the grassland source into the arable sink. However, excrements produced while the livestock was grazing during day time were lost for the arable land. As soon as it became possible to harvest hay and transport it to farmers' settlements, livestock could be kept in stables during wintertime increasing the catched manure which was distributed on the arable land. Acting like this, grasslands became more and more depleted in nutrients. In the meantime rules were prescribed in order to restrict the stocking densities hence safeguarding the sustainability of the system, inspiring Hardin (1968) to its famous article "The tragedy of the commons".

At the end of the 16th century, farmers on poor sandy soils in Flanders started to grow fodder crops both as catch crops after the harvest of the cereals and crops on the fallow land. The latter was quite a remarkable evolutionary step in the agricultural development since previously the fallow land was considered as a necessary part of the rotation in order to get rid of most of the weeds and to allow the release of newly available nutrients from the mother rock. Catch crops (as turnips) prevented leaching of valuable nutrients during late autumn and wintertime. The main forage crop grown on the fallow land was the nitrogen-fixing red clover, offering two main advantages: the fallow land produced an important quantity of forage and the clover left nitrogen for the subsequent cereals. Both the catch crops and the red clover allowed to increase livestock numbers substantially and hence the manure production. These developments allowed doubling the cereal yields, from an average of 1 ton/ha up to 2 tons/ha.

These developments are very well documented in Weston (1650), Mazoyer and Roudart (2002) and Hirata (2004).

A peculiar source is the work of Kjaergaard (2003, 2006), documenting the ecohistory of Denmark which can be considered as a pars pro toto for large parts of Europe. Kjaergaard describes how red clover (and later on coal) saved mankind from an ecological disaster. The strongly growing population as well as the political and military developments from the 16th century onwards urged many countries to rapidly cut their forests in a never ending need for construction material and fuel. As a consequence land degradation occurred which was a huge adversity in the continuous search for more agricultural land. The introduction of red clover cultivation and the accompanying livestock development allowed to restore soil fertility and food production as described above. The role of red clover was so admired that during the 18th century more than half of the agricultural land was covered with red clover in parts of Denmark. This practice became unsustainable and most likely due to pests and diseases the acreage of red clover started to decline. Fortunately at that time the use of coal as an energy source was introduced. In the light of current

feverish quests for renewable energy it is nice to know that the unwise depletion of renewable sources brought us close to an ecological disaster, from which we were saved by the use of two new sources: fossil fuels and leguminous crops. Currently one of the savers of the olden days threatens mankind while the role of the second one certainly is not finished yet.

2.2 The Main Source: Forage Production

All over the world large areas of grassland produce large amounts of forage for ruminants. In many cases, the use as grassland is the most sustainable use in areas with unfavourable climatic and/or geographic conditions.

After World War II, the use of grassland was intensified in many parts of Europe with fertilizer nitrogen as the main driver. Intensification was and is highest in the lowland areas. During the second half of the 20th century, high stocking rates combined with the use of high amounts of fertilizer nitrogen and concentrates caused environmental problems until present days. Modern grassland management has to cope with these and other challenges as described in Section 4.

2.3 Grassland as an Energy Crop

Grassland biomass can be used as a renewable source of energy. The biomass can be combusted or converted into biofuel or digested into biogas. Grassland management for biofuel production can vary from a very extensive use of semi-natural grassland to an intensive biomass production with C_3 and C_4 grass species. Tillman et al. (2006) argue that a floristic diverse prairie vegetation in the USA has a beneficial output/input energy ratio, since the energy input is very low. Swards are not fertilized, the biomass production is low and the nitrogen input comes from legumes. The European idea to produce energy grass with *Lolium multiflorum, Phleum pratense, Festuca arundinacea* or *Miscanthus* (e.g. *Miscanthus* × *giganteus*) on fertile land aims at a high biomass production with an accordingly high energy content. However, in these circumstances the energy input is quite high (predominantly by nitrogen fertilizers) and the output/input energy ratio may be relatively low. Ceotto (2007) is very critical in his review on grasslands for bioenergy production particularly when the biomass is combusted. In order to achieve a reasonable biomass yield one needs nitrogen (biologically fixed or fertilizer nitrogen). The combustion of a "nitrogen-rich" biomass releases nitrogen oxides; they may form ozone, which is a greenhouse gas. Whatever the management may be, recycling or adding nutrients is necessary to keep soil fertility at an acceptable level. In the absence of this, yields drop dramatically as is known from long-term grassland experiments as the Park Grass experiment in Rothamsted, UK.

2.4 Grassland and Ecosystem Services

2.4.1 Biodiversity

Reidsma et al. (2006) calculated a dose–effect relationship of the intensity of agricultural land use on biodiversity in the EU and concluded that grassland has the best ecosystem quality of all possible agricultural production systems. Biodiversity was expressed as the mean abundance of species originally present in natural ecosystems relative to their abundance in undisturbed situations and this was called ecosystem quality. In this respect, extensive grassland is at the top, but even intensively managed grassland has an ecosystem quality that is at least twice as high as the ecosystem quality of comparably managed arable land.

There is an overwhelming body of literature showing that *agriculturally used* grassland areas contain a high floristic and faunistic diversity; the less intensive the management, the higher the biodiversity. However, pressure on grassland habitats is steadily increasing although legislative measures and supporting schemes in the EU refrain this evolution. This pressure may change both species richness and species abundance causing a decline of the structural diversity of grassland. A change in botanical diversity, even in intensive managed grassland at low species number, may have important agronomic consequences, since in most instances the larger the botanic diversity, the higher the potential productivity (e.g. Kirwan et al., 2007).

Preserving and protecting semi-natural grassland really is essential since it has been demonstrated that it is very difficult to bring back botanical diversity by extensifying grasslands that previously had been managed intensively.

The botanical richness of the European grasslands is highly esteemed by grass and forage breeders (see Chapter 2, Section 3) who continue to collect genetic resources in order to improve grassland productivity and quality.

Recently in a number of European countries, water levels are increased in historically wet grasslands in order to promote a wet land flora and fauna (Grevilliot et al., 1998). In some of these areas grazing is needed to sustain the floristic diversity. However, parasites like liver fluke and poisonous plant species like *Senecio aquaticus* are a serious threat for animal health (Davis, 2005).

Less studied is the belowground biodiversity. Van Eekeren et al. (2007) give an overview of soil food web and the role of belowground biota and demonstrate that herbivorous nematodes and active microbial biomass (in particular bacterial biomass) are much higher in permanent grassland than in temporary grassland or in arable land. Earthworm abundance is about three times higher in grassland than in arable land; the abundance is significantly higher in grass–white clover mixtures than in pure grass stands. Upon ploughing down grassland, the earthworm abundance falls down very steeply but there is a remarkable and fast resilience when arable land is turned into grassland again (Van Eekeren et al., 2008).

2.4.2 Water Storage and Water Quality

Benoit and Simon (2004) studied the effect of grassland both on the hydrological cycle and on water quality. Compared to arable land, grassland generally has higher

water infiltration rates, preventing top soil loss and runoff. The older the grassland, the higher the infiltration capacity, owing to a better soil structure, more earthworm burrows and a higher organic matter content. Heavy trampling in grazed grassland may reduce infiltration capacity with 50%.

There is ample literature indicating that nitrate concentrations are very low under *cut* grassland (e.g. Nevens and Reheul, 2003a).

In a lysimeter experiment conducted in Belgium during more than 20 years (1972–1994), annual nitrogen leaching never exceeded 13 kg ha^{-1} y^{-1}. Swards of pure *Lolium perenne* were established on different soil types (irrigated and non-irrigated sand, sandy loam, loam and clay) and cut in a simulating grazing management at nitrogen dressings varying from 390 to 397 kg ha^{-1} y^{-1}.

According to Benoit and Simon (2004) summarizing data from different European countries, nitrate leaching in *grazed* grassland tends to be less than 50 kg N ha^{-1} y^{-1} as long as nitrogen fertilization is below 300 kg N ha^{-1} y^{-1}. The authors claim that respecting the EU Nitrate Directive (water should contain less than 50 mg NO_3^- l^{-1}) offers no problem, provided that the annual number of LU (livestock units) × grazing days is lower than 500.

Grasslands are rarely vulnerable to groundwater contamination by pesticides. Microbial contamination of surface water may occur if livestock enters ditches to drink and drops its excrements and urine directly in the water.

2.4.3 Landscape and Ecotourism

In the lowland, citizens appreciate grazing animals in the landscape, for emotional reasons and because they associate grazing with animal welfare. In the highland areas and some semi-arid areas (e.g. the Iberian Dehesa ecosystem) grassland associated with grazing animals promotes ecotourism, offering an extra income for the inhabitants.

3 Grassland as a Sink: Carbon Storage

Grasslands harbour approximately 34% of the global stock of carbon in terrestrial ecosystems, while forests store about 39% and arable ecosystems approximately 17% (Pedro Silva et al., 2008). While in forests the carbon predominantly is stored in the vegetation, most of the grassland carbon stocks are in the soil. Carbon enters into grassland soil through litter fall, root turnover and carbon exudation from roots. It is released from the soil by respiration and by leaching.

Mestdagh et al. (2004) surveyed a large number of grasslands in Belgium and concluded that permanent grassland contains about 50% more soil organic carbon (SOC) than temporary grassland and grazed grassland contains about 50% more SOC than cut grassland: in both cases approximately 150 vs. 100 Mg ha^{-1} in a soil profile of 60 cm depth. The latter trend was confirmed by data of Casals et al. (2004) reporting results from the subalpine and alpine grasslands in the Pyrenees.

Absolute quantities of SOC vary according to climate (temperature, precipitation), soil type and the time scale of particular managements. In a Belgian experiment (Bommelé, 2007; Van Eekeren et al., 2008), comparing 36 years of permanent arable land with 36 years of permanent grazed grassland on a sandy loam soil with N dressings on the grassland varying between 230 and 350 kg ha^{-1} y^{-1}), 35 Mg ha^{-1} C was found in the arable land, vs. 55 Mg ha^{-1} in permanent grassland (depth of the soil profile: 30 cm). Averaged over 36 years, this means an increase of 0.57 Mg ha^{-1} y^{-1}, which is very close to the amount of 0.52 Mg ha^{-1} y^{-1} cited in Soussana and Lüscher (2007).

Hopkins and Del Prado (2006), reporting on carbon balances in North American Great Plains, argue that a grassland sward aged more than 20 years, no longer acts as a carbon sink. The situation is probably similar in the European temperate climate as indicated in the Park Grass Experiment in Rothamsted (Johnston, 1986). The latter experiment also indicates that upon ploughing down the grassland, the breakdown of SOC occurs nearly twice as fast as its accumulation during the grassland phase.

The concentration of CO_2 in the air is expected to influence plant growth and carbon turnover. Long-lasting Swiss and German experiments indicate that an elevated CO_2 concentration had little effect on the carbon balance in cut swards of either *L. perenne* or *Trifolium repens*. The extra biomass production owing to the elevated CO_2 concentration was to a large extent neutralized by the ecosystem respiration (plant+soil microbiota).

The silvopasture type of agroforestry may store an additional amount of carbon, both below and above ground. However, few quantitative data are available.

4 Modern Grassland Management: The Combination of Source and Sink in a Tempting Exercise in Sustainability

4.1 Grazing

Modern grassland management tries to optimize the source and the sink function of grassland, in order to fit into the sustainability framework. Modern grassland management is "making sustainability at work". Taking care of not losing the link between livestock and land is a prerequisite (Naylor et al., 2005). Reading the following paragraphs one should remember that sustainability tries to reconcile three pillars: ecology, economy and social aspects. They may act in a concerted way, but in some cases their reconciliation is not easy.

The role of grassland in livestock farming in Europe is connected to both socio-economic and natural conditions. Pflimlin and Todorov (2003) divide Europe into different regions according to geographic and climatic characteristics. The hilly, mountainous, Mediterranean and Nordic regions are confronted with unfavourable soil and climatic conditions and apply on average a rather extensive livestock farming (mainly meat production), with a dominating role for grazing. The link between land and livestock is conserved well. Eco-efficiency, defined as "more (economic)

value with less environmental impact", is quite high owing to a rather small denominator: low use of sources and low pressure on sinks. If well managed these farming systems may take advantage of "the ecology of scale" as defined and demonstrated by Schlich et al. (2006). The adoption of agri-environment programmes is quite high, offering good opportunities to safeguard ecosystem services and biodiversity in particular. In many cases the agri-environment programmes do save these grasslands from abandonment. Abandoned grassland is likely to evolve into a natural forest in large parts of Europe. In the short term this evolution is expected to lead to a decline in biodiversity. The patchy landscape will close into a monotone view. Ecotourism will decline and without the buffering capacity of the grasslands, the risk on large forest fires is expected to increase.

In north-west Europe, intensive dairy farming is driven by a combination of grassland, forage crops and supplementation with concentrates. During the previous decades, the role of grassland was diminishing constantly, while the use of forage maize and concentrates was increasing steadily. Reasons for this evolution are connected mainly to animal aspects (highly demanding and balanced feed rations) as well as to socio-economic aspects. The economy of scale becomes more and more important to survive for dairy farms in areas with intensive production systems. Large herds fed with feed of constant quality and milking robots are components of these trends. Both output and input are high as well as pressure on sources and sinks, jeopardizing eco-efficiency and the link between land and livestock.

Van den Pol-van Dasselaar et al. (2008) present an excellent overview of current developments. The authors argue that grazing must be able to produce high-quality forage within the environmental constraints, at a low cost, with a high labour efficiency and an acceptable comfort for the farmer (representing the three pillars of sustainability). The larger the herds, the more difficult it becomes to manage grazing within these borderlines and the more attractive it becomes to apply a "cutting only" management: sward productivity is higher (owing to less frequent defoliation) and manure can be spread more evenly compared to the droppings of the animals in a grazing situation. Compared to grazing, residual mineral soil nitrogen at the end of the growing season is much lower under cutting.

From an economic point of view, grazed grassland continues to be the cheapest forage, but it takes good managerial skills to produce and to offer a constant high quality of grazed grass owing to physiological and phenological reasons. Indeed, the key factor in grazing is a high allowance of highly digestible forage (see Figure 1). If this target is reached, differences between rotational and continuous grazing are small for a given stocking density. However, owing to the continuous defoliation, continuously grazed swards have a less developed root system, making them more vulnerable to summer drought. Decruyenaere et al. (2007) report higher residual soil nitrate in continuous grazing compared to rotational grazing.

Allowances for optimal animal performance are reached with sward heights of approximately 10 cm in continuous grazing systems (Peyreaud et al., 2004; Delagarde et al., 2001). The relationship between daily herbage intake (DHI) and daily herbage allowance (DHA) has been studied in detail in France. Under rotational grazing DHI reaches a plateau at a DHA of approximately 60 kg DM day^{-1},

Fig. 1 A high allowance in a rather short sward is optimal for animal performance. Tolerance of cultivars to cattle grazing under these conditions is tested as part of some VCU schemes such as in the UK (Photo T. Gilliland)

as given by the formula of Delagarde et al. (2004): DHI = $18.4(1–e^{-0.0466\,DHA})$, meaning that the animals graze only a fraction of what they need. Offering a long sward (which offers theoretically an easy bite) to achieve this target is not an option, since large parts of such a sward remain uneaten, deteriorating sward structure and herbage quality as the growing season progresses. The compromise lies in offering a high allowance in a rather short sward: approximately 2000 kg DM ha^{-1} above the grazeable height of 5 cm. The more leafy the sward is underneath, the higher the intake. Compared to pure grass swards, intake is higher in mixed swards, which might be an incentive to use mixtures of, e.g. ryegrasses mixed with white clover. The higher intake is probably due to a faster rate of particle breakdown of the legume in the rumen, associated with a faster rumen clearance.

Disrupting the grazing period stimulates forage intake. A good strategy is to take advantage of post-milking motivation to graze, e.g. by allowing the animals to graze for no longer than 4 h after each milking. Shorter grazing periods offer an extra advantage: the high crude protein content of the leafy grass can be supplemented in the cow shed with concentrates or with a forage with a high energy value. Acting like this, the nitrogen efficiency of the animal (NUE) increases. Combined with an enhanced NUE in the grass plants, the overall efficiency of the nitrogen use may be improved.

Extra arguments pleading for grazing in dairy farming refer to animal welfare (e.g. less lameness, better udder health and better fertility) and to the compositional analysis of animal products. There is an ample body of literature indicating a favourable effect of fresh forage on the fatty acid composition of milk and meat: the more fresh forage the animals ingest (and the more botanical diverse

the vegetation) the higher the concentrations of beneficial polyunsaturated fatty acids and conjugated linoleic acid in milk and intramuscular fat (e.g. Elgersma et al., 2006).

Combining cattle and sheep grazing improves herbage utilization and grazing productivity, since sheep consume the refusals of the cattle (Nolan et al., 1999). However, residual soil nitrate in the autumn is higher in a system of mixed grazing. Mixed animal type grazing has another advantage: different animal species act as cleansers for one another's parasites, owing to a high host specificity of lungworms and gutworms.

4.2 Opportunities for Temporary Grassland

4.2.1 Ley–Arable Rotations

In ley–arable rotations, short-term grassland rotates with an arable period. The diversity in grass–arable rotations in Europe is described in detail in Conijn et al. (2002).

Ley–arable rotations are an essential part of the organic farming systems in order to accumulate soil organic matter and to provide nutrients to the following crops, as well as to help control weeds and diseases. The sward usually is a grass–leguminous plant mixture. In mixed farming types the forage is used on farm, in stockless organic farming systems the forage produced in leys with a leguminous component may be sold to mixed farms, or the vegetation may be cut several times a year and left as a mulch on top of the soil; in the latter case nutrients are continuously recycled.

Ley–arable farming brings along extra costs for seed, seed bed preparations and fences and drinking facilities during the grassland stage. Owing to the short duration of the grassland, floristic diversity has no high priority. Ploughing stimulates mineralization of soil organic matter, producing extra CO_2 emission. The potential strength of a ley–arable system lies in (a) the release of nutrients (accumulated during the grassland stage) to the arable crops (allowing to save on, e.g. nitrogen), (b) in the improvement of soil structure and (c) in a potential higher yield of the grassland on the longer term, since the reseeding may take advantage of the newest cultivars and since rooting systems of young grassland usually penetrate deeper soil layers making the vegetation less prone to drought stress.

Nevens and Reheul (2002, 2003b) and Reheul et al. (2007) studied ley–arable farming for over 30 years on a sandy loam soil in Belgium. Periods of 3 years of temporary grazed grassland (fertilized with 230–350 kg N ha^{-1} y^{-1}) were followed by periods of 3 years of arable crops.

Net energy for lactation (NEL), measured by monitoring live weight gain of heifers, in temperate grassland did not outyield significantly values recorded in permanent grassland over a period of 36 years which was surprising, since every new cycle of temporary grassland was sown with the newest highly productive varieties.

Ploughed down temporary grassland had a nitrogen fertilizer replacement value (NFRV) of about 250 kg nitrogen ha^{-1}: ± 50% of it during year 1, ± 30% during year 2 and ± 20% during year 3. The crop that opened the arable period did not need any nitrogen to give an economically optimum harvest, whether the crop was forage maize, fodder beet or potato.

Compared to permanent grassland and permanent arable land, the ley–arable system had an intermediate position in the build-up of soil organic matter, soil organic carbon and soil organic nitrogen.

In the absence of grazing, soil N accumulation is estimated to be lower owing to a much higher export of N. How much lower is not well documented, although the results of Hansen et al. (2005) suggest that the residual N effect was only 13% lower.

To ensure an efficient use of the high amounts of mineralized nitrogen immediately after the destruction of the grass sward, the destruction should occur during spring time and the opening crop should be a nitrogen greedy crop. In the absence of the latter, a catch crop should be installed after the harvest of the main crop in order to minimize the risk of high residual soil N concentrations.

An excellent review on nitrogen dynamics and carbon cycling in ley–arable rotations is given by Vertès et al. (2007).

4.2.2 Frequently Renewed Grass Swards

In line with data presented in previous paragraphs, the message is clear: permanent grassland with a good botanical composition should not be renewed.

If by whatever reason resowing is necessary, the establishment of new grass swards in previously cropped arable land is a better option than ploughing up the sward followed by reseeding as documented by Reheul et al. (2007). Newly established grassland outyielded old permanent grassland spectacularly during a very dry summer owing to its deeper rooting system. However, the yield bonus of new swards usually fades away after 4 years.

The initial abundance of white clover is much higher when grass–white clover is established in arable land. This superior abundance continues for several years.

Residual soil nitrogen stayed below 50 kg NO_3^-–N ha^{-1} in a soil profile of 0–90 cm, even when up to 300 kg N ha^{-1} y^{-1} was provided to the newly established sward and this was not needed to get a full harvest during the year of establishment.

5 Conclusion

Grassland in Europe is developing differently according to geographic and climatic differences. The balance between socio-economy and ecology is hard to reach and to maintain. In less favoured areas socio-economy is the main problem, while in zones with intensive use of grassland, the care for the ecology is cumbersome. Permanent grassland should be managed very carefully, since it may cope with the pillars of

sustainability in the most balanced way by its reasonable yields and its high potential for floristic diversity and other ecosystem services. Ley farming probably has a higher yield potential, but it may be weaker from an ecosystem services' point of view.

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Genetic Resources

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1 Introduction

Plant genetic resources (PGR) for food and agriculture consist of the diversity of genetic material contained in traditional varieties and modern cultivars grown by farmers as well as crop wild relatives and other wild plant species that can be used for food, feed for domestic animals, fiber, clothing, shelter, wood, timber, energy, etc. (FAO 1997). Fodder crop genetic resources broaden the FAO definition of PGR, which is based upon field crops. In maize and many other crop species, the wild form of the cultivated species no longer exists, since breeding for domesticity has resulted in plant species being unable to reproduce without the helpful hand of humans. Forages are less domesticated (Harlan 1983). Unlike many field crops, wild forms of common forage species still exist, as well as feral (naturalized) forms (populations that originated from forage crops, but that escaped to persist in the natural environment). Such wild populations are usually called "semi-natural" because they have developed in an agricultural situation, but without conscious selection. They would not fall in any of the categories of the definition of PGR as cited above but can be regarded similar to crop wild relatives. The closeness of wild and cultivated forms of fodder crop species makes a wealth of natural genetic variation readily accessible for use in breeding.

Evidently, PGR are indispensable for any breeding effort. At first and very obviously, PGR with desirable traits must be chosen to initiate the breeding process. The choice of this initial material is crucial for the programme because breeding is a long-lasting process, and many years of selection and recombination are needed before success can be assessed and finally, a new variety can be created. How much the origin of the starting material can influence the properties of a breeding programme has been extensively studied in an interesting example from New Zealand (Bahmani et al. 2001). Recent varieties derived since 1975 from the "Mangere" ecotype of perennial ryegrass differed fundamentally in yield potential, growth habit and behaviour under grazing from older varieties derived from the

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geographically distinct "Hawke's Bay" ecotype, which had previously dominated the New Zealand ryegrass seed market between 1936 and 1964. While the more recent varieties derived from the "Mangere" ecotype were higher yielding under cutting, partly due to a more erect growth habit and a greater proportion of fertile tillers, they were less adapted to grazing than the older varieties derived from the "Hawke's Bay" ecotype, leading to problems of persistence. It is interesting to note that this fundamental difference was only discovered after release of the more modern varieties. This observation points to the fact that the best possible knowledge of the properties of potential starting materials should be obtained before making the choice.

Second and less obviously, PGR are needed to add new variability to an existing breeding programme. A basic tenet of plant breeding is that gain from selection increases with an increase in additive genetic variance for a given character (Fehr 1987). Selection inevitably decreases additive genetic variance in breeding material. For example, when we select for disease resistance, we aim at eliminating susceptibility genes in order to obtain a population which is homozygous for major resistance genes. This resulting population has a high level of resistance and low variability in susceptibility to the disease. However, since we select only a limited number of individuals in each cycle, genetic diversity for all other traits is also affected. Rare alleles are rapidly lost and opportunities for selection decrease because the most frequent alleles become fixed in the population. Furthermore, in out crossing self-incompatible taxa, inbreeding depression may occur because there is a higher chance of homozygosity for deleterious recessive genes.

An infusion of exotic germplasm at this point will increase additive genetic variance. However, there will be a reduction in mean performance, as the population moves away from the selective peak reached through previous breeding efforts. The less adapted the PGR, the greater the drop in performance. With continuous selection within the broadened breeding population, performance will improve again. However, if introduced beneficial alleles are the same as those already present in the breeding population, further selection will return the population only back to the same selective peak, and no net gain in performance will be realized. Only if the introduced alleles are unique, can selection increase the level of performance to a higher selective peak. Therefore, PGR most likely to improve upon quantitative traits will be those accessions that possess favourable alleles not present in the breeding gene pool. Humphreys (2003) reviewed criteria and objectives of the use of new genetic material in a long-term breeding programme with a special focus on sustainability. Appropriate strategies for the use of PGR in the breeding programme will be discussed in Section 5 of this Chapter.

2 Types of Genetic Resources and Conservation Modes

2.1 Categories of PGR

Four basic categories are of potential importance for fodder crop and amenity grasses breeding programmes:

1. Wild relatives: Most fodder crops and amenity grasses belong to large genera with several more closely related species of potential interest in breeding. However, due to their relatively young history as crops, available genetic variability within the cultivated species of fodder crops and amenity grasses is generally still quite large. Nevertheless, the use of wild relatives in breeding programmes is of importance in allopolyploid species with complex systematic like alfalfa or white clover, and wild relatives have been used successfully to introgress specific characters into the cultivated species, such as the profuse flowering trait from *Trifolium nigrescens* into white clover (Marshall et al. 2008).
2. Wild and semi-natural forms of cultivated species: These two sub-categories are difficult to distinguish for most species because there is no clear borderline between wild and semi-natural forms. This is because permanent grassland in most relevant cases exists only as a consequence of human agricultural activity in zones where forests would be the natural vegetation. Adapted native grasses originating from non-agricultural habitats settle in permanent grassland together with naturalized populations of the same species which may have spread from an initial seeding. Therefore, such populations form a continuum from wild populations in non-agricultural habitats to populations of natural and semi-natural grassland. Rather than trying to assign them to an either wild or semi-natural origin, it is more appropriate to address such populations as *ecotypes*. Using the term "ecotype" implies populations which have adapted to a known environment after many years of natural selection, usually involving natural re-seeding but without deliberate human interference such as selection, seed harvest, or human-mediated seeding. For ecotypes, natural selection is the main driving force of genetic differentiation. Ecotype populations usually do not arise from an initial sowing of the species, neither sown as such nor as part of a seed mixture, but from spontaneous seedlings emerging gradually over the years through natural spreading. That is, human interference in the development of an ecotype is limited to actions of usual management practices, such as frequency and type of utilization, or intensity of fertilization. If sufficient cycles of recombination with local genetic material and natural selection have occurred, an initial sowing of a fodder crop variety may also give rise to an ecotype population. In ecotype studies, it is often postulated that a certain number of years must have elapsed since the last deliberate re-seeding before a population can be called an ecotype. The time span postulated ranks from 10 to 25 years. Examples of ecotype studies with relevance to fodder crops and amenity grasses breeding are discussed in Section 3.1.
3. Landraces: Populations which have adapted to a specific region or location, such as a farm (farm varieties, "Hofsorten") by repeated seed harvest and human-mediated re-seeding in the same region or location. The term "landrace" implies that human interference plays an important role in the development of the population. In the case of landraces, human actions are usually carried out deliberately to improve local adaptation, e.g. by re-seeding the surfaces with locally produced seed, and by carrying out seed harvest after several years of utilization as forage to improve persistency. Prominent examples of highly valuable, traditional landraces are alfalfa in Italy (Torricelli et al. 2003), timothy in Norway (Schjelderup

et al. 1994), and red clover in Switzerland (Boller et al. 2003; Hermann et al. 2003; Kölliker et al. 2003).

4. Varieties: Any cultivated variety (cultivar), whether freely available on the market, protected by plant breeder's rights, or having become obsolete and stored in gene banks, can be used in breeding without any restriction. The right to freely use even protected varieties as PGR in breeding is called "breeder's exemption" and is an important provision of the international convention for the protection of new varieties of plants (UPOV 1991). For use in breeding, varieties have the advantage of being precisely described through the registration procedure for distinctness, uniformity and stability (DUS), and usually have been evaluated extensively in official tests for their value for cultivation and use (VCU). Furthermore, commercially successful varieties have proven their ability to give satisfactory seed yields. These properties render cultivars very popular as PGR in fodder crop breeding. Once a variety has ceased being produced for the market, and thus has become "obsolete", an appropriate seed sample is added to the gene bank collection of the respective country. The gene bank will then assume from the breeder the responsibility for long-term maintenance of the variety's integrity, and for securing availability of seed for use in breeding or research. During the commercial lifetime of a variety, seed samples can easily be obtained from the breeders who exchange their varieties free of charge as a voluntary service to their peers.

2.2 Modes of PGR Conservation

Two modes of conservation are of importance for fodder crop and amenity grasses PGR: While all types of PGR are maintained *ex situ* as seed samples in gene banks, wild relatives, ecotypes and landraces can also be maintained *in situ* (referred to as "on farm" in the case of landraces). The two approaches differ fundamentally in their objectives regarding the genetic make-up of PGR. In *ex situ* conservation, maintaining the genetic integrity of the original seed sample is a major concern and all measures of collection, storage, regeneration, and distribution aim at keeping presence and frequency of alleles within the population as constant as possible. Conversely, the objective of *in situ* conservation is to maintain the environment which has allowed the development of the distinctive properties of the PGR. In the case of *in situ* conservation, genetic evolution is deliberately made possible in order to allow a further development of PGR to even better match the requirements of their specific environment. The common objective of the two strategies is to conserve a maximum of different alleles and the largest possible amount of genotypic diversity with as few individuals as possible (Hayward and Sackville Hamilton 1997).

3 Genetic Resources Maintained *In Situ*

3.1 Breeding Importance of *In Situ* Germplasm

Historically, genetic resources growing *in situ* have been by far the most important sources of germplasm used in breeding of fodder and amenity grasses and most

perennial legumes, with the exception of alfalfa and red clover which have a longer tradition of being cultivated as sown crops. In the search for well adapted and persistent genetic materials, breeders have systematically explored permanent grassland in their target regions to collect ecotypes. They followed the recommendation of Hertzsch (1959) that "suitable starting plants will be found on old permanent grassland with an association of species which is typical for the respective situation". His discussion about the starting material for grass breeding clearly pointed to the great potential value of diverse natural permanent meadows and pastures as reservoirs of well-adapted populations of grassland species. Undoubtedly, the use of adapted genetic material collected in permanent grassland has been of great benefit to early fodder crop breeding. It has dramatically improved persistency of fodder grasses compared to the often exotic provenances of grass seed that had been used previously. The bulletin of perennial forage plants listed in the French national catalogue in 1984 (I.N.R.A. 1984) lists ecotypes as the material of origin for the large majority of varieties for which an unequivocal origin was declared (Table 1).

Table 1 Declared origin of varieties in the official French catalogue of fodder plant varieties accepted 1957–1984 (I.N.R.A. 1984)

	Number of varieties originating from:			
Species	Ecotypes	Varieties or landraces	Ecotypes and varieties	Breeding material of diffuse origin
Dactylis glomerata	12	0	0	1
Festuca arundinacea	11	0	0	6
Festuca pratensis	4	1	1	1
Phleum pratense	5	0	3	1
Lolium perenne	10	1	8	9
Lolium multiflorum ssp. *italicum*	5	1	3	10
Total	47	3	15	28

Nowadays, breeders rely much more on crosses between varieties as starting materials for their breeding, rather than introducing newly collected material. However, since most of the older varieties have been derived from an original collection in grassland, we can assume that the majority of varieties currently in use trace back at least partly to breeding material originally created from collections of PGR *in situ*. This is reflected by the small genetic distance between cultivars and ecotypes found in molecular studies, for example in perennial ryegrass (Bolaric et al. 2005; McGrath et al. 2007), meadow fescue and Italian ryegrass (Peter-Schmid et al. 2008b).

In modern forage crop and amenity grasses breeding, collected material of ecotypes still keeps its significance as a source of hitherto unused genetic variation for particular traits of interest. For example, Beuselinck (2004) used material of wild accessions from Morocco to introduce the rhizomatous growth character into *Lotus corniculatus*. Swiss ecotypes of *Lolium multiflorum* were used successfully to create a variety with improved early spring growth and resistance to snow mould diseases, and a variety which combines a strong tendency to form inflorescences in the seeding year with excellent persistence (Boller et al. 2005a).

3.2 Grassland-Dominated Regions as Centres of Diversity

Grassland-dominated regions provide the most diverse opportunities for collecting PGR of fodder crops and amenity grasses *in situ*. For most temperate species of interest, non-irrigated permanent grasslands are concentrated in zones with at least 800 mm of annual rainfall and about 9°C annual mean temperature. This is particularly true for more intensively utilized grassland. Areas suitable for intensive agriculture in zones with 600–800 mm of annual rainfall would still provide good conditions for permanent grassland, however, such land was turned into arable land wherever possible. If livestock is reared in these zones, temporary grassland and arable forage crops provide the main part of feed rather than natural grassland.

Grassland-dominated regions occur in temperate zones around the world. For example, northern Africa, temperate Asia, as well as the Atlantic islands of Macaronesia belong to the area of natural distribution of important grassland species. In Europe, temperate grassland-dominated regions are found in the Pyrénées, on the British Isles and the Balkan, but are particularly diverse in the zone between the northern foothill of the Alps and the coastal zones of the Atlantic and Baltic sea (Figure 1). In the following discussion, we will focus on Europe from the Alps northwards as one of the major centres of diversity of fodder crops and amenity grasses. Most temperate grassland species are truly indigenous to that region, while some most likely have developed naturalized populations from historic introduction as a fodder crop. Scholz (1975) suggested the latter status for *Arrhenatherum elatius, Alopecurus pratensis* and *Phleum pratense* for the whole of Europe, and also for *Lolium perenne, L. multiflorum* and *Trisetum flavescens* for many regions where these species are restricted to man-made habitats.

Apart from climatic and edaphic factors, grassland management contributes substantially to the diversity of grassland plant communities. Intensive management, specifically a high grazing pressure, tends to decrease species diversity. In recent years, targeted programmes aiming at a more relaxed grassland management as part of agri-environment measures have been established to increase biodiversity. However, the response of the plant communities to reduced management intensity is slow (Marriott et al. 2004). Nösberger et al. (1998) concluded that management for habitat heterogeneity at all scales will conserve most of the biotic diversity at a site. Such a system considers diversity not only on a small scale of cutting or fertilization regimes of a particular field, but includes the larger scale of landscape

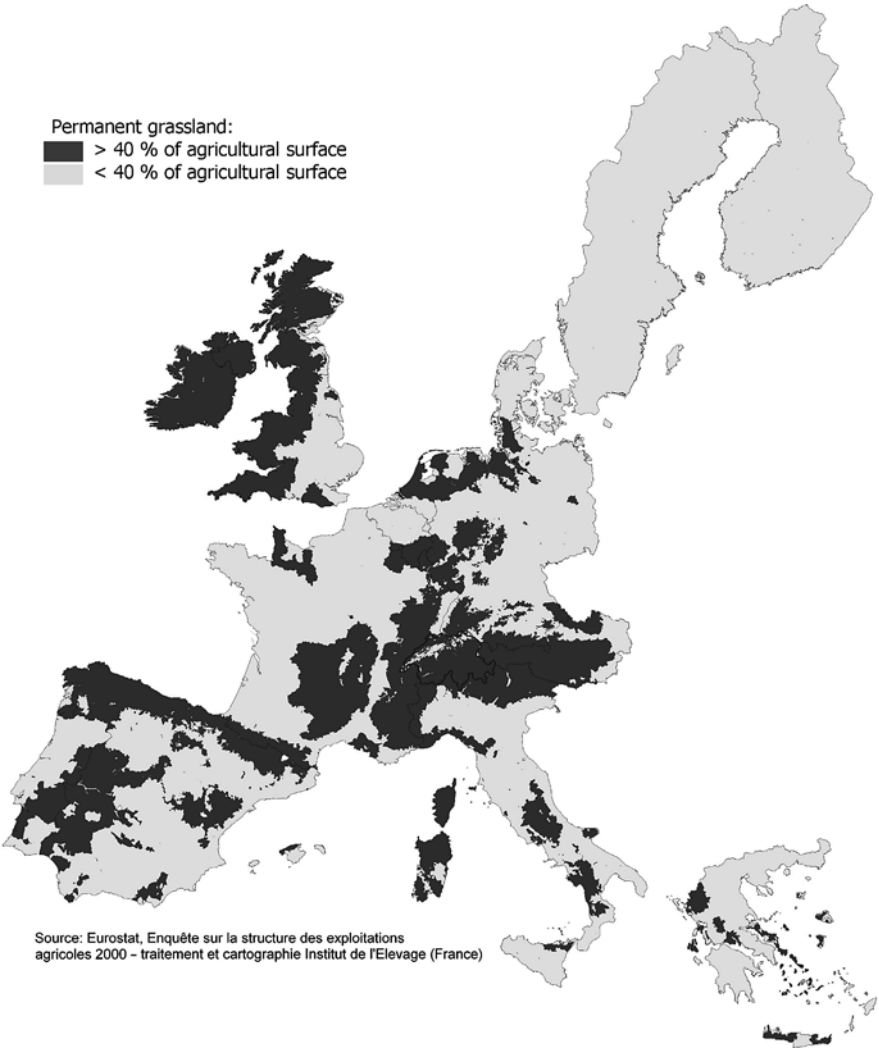
Permanent grassland:
> 40 % of agricultural surface
< 40 % of agricultural surface
Source: Eurostat, Enquête sur la structure des exploitations agricoles 2000 – traitement et cartographie Institut de l'Elevage (France)

Fig. 1 Regions of Europe with important proportions of permanent grassland per agricultural surface. Adopted from Pflimlin et al. (2005); Swiss data supplemented by E. Szerencsits

structure. It will allow for intensive management of the most favourable pastures to produce high-quality ruminant feed, along with maintenance of infrequently cut hay meadows providing opportunities for environmental services (Nösberger and Rodriguez 1998). Clearly, maintaining diverse grassland on the landscape scale in such a way will also assist *in situ* conservation of PGR of fodder crops and amenity grasses.

3.3 Criteria and Strategies for Collecting PGR *In Situ*

Depending on breeding objectives, two basic criteria need to be considered in making a strategy for collecting fodder crops PGR *in situ*:

1. When the primary objective is to enlarge genetic diversity of the breeding programme, the relative degree of genetic variation within and among sampling sites will affect decisions about the number of sites to be visited and the number of individuals to sample per site.
2. When the objective is to find new genes affecting particular characters, the choice of collecting sites will be influenced by the presence of environmental or management factors that are selective forces for traits of interest.

Genetic variation within and among ecotype populations has been studied using molecular markers for a number of grassland species (Table 2). Although different marker systems have been used to assess diversity and this may affect the estimates, a general picture emerges from these studies showing that the variation within populations accounts for at least 60 and up to 98% of the total genetic variation. This suggests that sampling a large number of sites with just a few individuals is less effective for capturing genetic diversity than sampling fewer sites with a higher number of individuals.

Table 2 Contribution of among and within populations variance in marker-based studies of genetic diversity of grassland ecotypes, based on analysis of molecular variance (AMOVA)

Source	Species	Marker system	No. of ecotype pops.	No. of indiv. per pop.	AMOVA: % of variance	
					Among pops.	Within pops.
Bolaric et al. (2005)	*Lolium perenne*	RAPD	22	20	29	71
Rudmann-Maurer et al. (2007)	*Poa alpina*	SSR	54	8	25	75
Reisch et al. (2003)	*Sesleria albicans*	RAPD	25	4	38	62
Peter-Schmid et al. (2008a)	*Lolium multiflorum*	SSR	12	23	2	98
	Festuca pratensis	SSR	12	23	4	96
McGrath et al. (2007)	*Lolium perenne*	cpSSR	61	16	37	63
Fjellheim et al. (2005)	*Festuca pratensis*	AFLP	15	20	31	69

Molecular studies published so far do not point to a specific strategy in the search for sites with a high genetic variability. In a long-term experiment at two locations, it was shown that genetic variation within ecotype populations of *Festuca pratensis* was negatively affected by a higher intensity of agricultural management, namely, by an increase of defoliation and fertilization frequency (Kölliker et al. 1998). However, this effect was not observed in a larger collection of ecotype populations of the same species (Peter-Schmid et al. 2008b). Rudmann-Maurer et al. (2007) did not find an effect of fertilization intensity on genetic diversity of *Poa alpina* ecotype populations, nor did they find a reduction of genetic diversity with decreasing abundance of the species in the sward.

However, molecular marker systems which are suitable to describe genetic diversity, such as RAPD, AFLP or SSR, deal with anonymous loci and are often located in non-coding regions of the genome. Although morpho-physiological characters are more cumbersome to assess and need replications in space and time to yield accurate results, they have been helpful in pointing to a specific strategy of sampling many sites to capture extremes in trait values. When the variability of morpho-physiological characters of a comparable set of ecotypes was assessed in relation to molecular marker variability, usually, a larger among populations variability was observed for the morphological characters than would have been expected from the variability of molecular markers. For example, Fjellheim et al. (2007) with Norwegian and Peter-Schmid et al. (2008b) with Swiss material found ecotypes of *F. pratensis* to exhibit larger ranges of mean values for 16 out of 19 and 13 out of 15 morphological characters investigated, respectively, than for cultivars, whereas the opposite was true for molecular marker diversity (Fjelllheim et al. 2005, Peter-Schmid et al. 2008a). This implies that sampling ecotype populations from a larger range of sites will increase the chance of including the extremes for the traits of interest.

The influence of environmental factors as selective forces at the genotypic level is at the base of the concept of ecotypic differentiation. It generally holds true that ecotypes from contrasting climates are better adapted to climatic conditions which are more similar to those at their origin. Winter hardiness and resistance to heat or drought are typical examples. Resistance to biotic stress can be expected in a climate which is favourable for the pathogen. A well-documented example is crown rust in ryegrasses and fescues which affects populations from high altitude much more strongly than populations from low altitude, where the pathogen *Puccinia coronata* finds better conditions for survival and spreading (e.g., Peter-Schmid et al. 2008b, Balfourier and Charmet 1991).

However, management factors can dramatically override the effects of environment and lead to strong differentiation on a small spatial scale. Tyler (1988) demonstrated this with an example of *L. perenne* sampled either within a hay meadow or on a path leading through that meadow. The population from the path flowered 30 days later, produced over five times less dry matter in spring, but suffered five times less from a freezing test than that from within the meadow. These differences were as large as the range observed in a more general way between southern and northern ecotypes from the whole of Europe. From this and other

similar examples, it can be concluded that collections should be made in zones with a climate similar to that of the target region of the breeding programme, but management history of the sites should be taken strongly into account.

An obvious way to infer management history of a potential sampling site is to assess floristic composition of the vegetation present. Plant species composition at a given moment is a good indicator of management factors which have been effective at the site over previous years. It is reasonable to assume that management factors affecting plant species composition will affect genetic differentiation of ecotypes in a similar way, and therefore, similar ecotypes of a species can be expected in grasslands of similar floristic composition or vegetation classification. However, this plausible correlation has not been studied to a great extent. A recent study with *L. multiflorum* (Boller et al. 2009) suggested that ecotypes from sites the vegetation of which was classified as *Lolietum multiflori* were more productive and showed better resistance against bacterial wilt and snow mould than ecotypes from *Arrhenatherion* sites. This suggests that the chances of finding well-adapted germplasm of a species can be increased by choosing collecting sites with a floristic composition pointing to agricultural management similar to that of the target use. Additionally, visiting places with contrasting floristic composition appears to be a good way to increase genetic diversity within a collection of a species.

3.4 Protection of PGR *In Situ*

During the past few decades, the protection of fodder crops and amenity grasses PGR maintained *in situ* has received increasing interest. This may be exemplified by two, temporally spaced reviews of PGR activities of the same institution, namely the former Welsh Plant Breeding Station in Aberystwyth (UK), nowadays the Institute of Biological, Environmental and Rural Sciences. While Tyler (1988) described naturally occurring ecotypes of forage grasses as "a seemingly limitless gene pool of variation on which the breeder can draw", Humphreys (2003) stated that "although it is diminishing year by year through genetic erosion, a wide range of valuable genetic resources are still available in the natural or semi-natural grasslands of Europe". He listed eight types of habitat risks which would justify collections to conserve adapted gene complexes, among which "ploughing and re-seeding" and "management change" were the two most important. The potential need of protection of forage grass populations as PGR is therefore seen in the same context as the protection of habitats to maintain biodiversity at the plant and animal species, as well as the ecosystem level, where general intensification of agricultural production is regarded the major threat to the conservation value of grassland (Marriott et al. 2004).

Nösberger (1994) presented an approach of promoting an individual farm-based grassland system with a varied, site-specific management supporting long-term, floristic stability of grassland as a promising way of encouraging farmers to contribute to the maintenance of biodiversity. On a landscape scale, such a system results in a diversity of habitats (see Figure 2) which creates opportunities for *in*

Fig. 2 Permanent grassland of varying intensity of management provides opportunities for *in situ* conservation in grassland-dominated regions such as in Northeastern Switzerland (Photo G. Brändle)

situ conservation of diverse populations of grassland species. Options to achieve both agricultural and nature conservation objectives in grassland systems were discussed in a similar way by Wilkins and Harvey (1994). The need for financial compensation to farmers for the economic constraints of such systems has been recognized and is implemented in modern agricultural policies, e.g. in the Common Agricultural Policy (CAP) of the European Union. Whether or not such an overall strategy is sufficient to adequately protect *in situ* conserved PGR remains open to question.

Programmes for promoting biodiversity with a focus on nature conservation concentrate on extensively managed, species-rich grassland. However, more intensively managed grassland may hold ecotypes which are of greater interest for future fodder crop breeding. Peter-Schmid et al. (2008a) showed that Swiss ecotype populations of *F. pratensis* from extensively managed habitats with a high nature conservation value contained significantly less rare alleles than populations from habitats managed more intensively. Management intensity also had a significant influence on morphological characters (Peter-Schmid et al. 2008b). Different conclusions were drawn by Van Treuren et al. (2005) for ecotypes of *L. perenne* and *Trifolium repens* from old Dutch grasslands. They concluded that no specific conservation measures were needed for ecotypes from pastures in agricultural use because they did not differ basically from ecotypes from nature conservation areas. This may reflect the absence of habitat fragmentation in Dutch grasslands, preventing strong ecotypic differentiation. In regions with a more variable agricultural landscape, the protection of fodder crop PGR in grassland of agricultural use with different levels of intensity appears to deserve adequate attention.

4 Genetic Resources Maintained *Ex Situ*

Ex situ germplasm collections serve a dual purpose. They are an important tool for conserving genetic diversity and they provide genetic resources for a broad range of users (Greene and Morris 2001). *Ex situ* conservation activities can be divided into the following general categories: acquisition, maintenance, evaluation and distribution.

4.1 Forage Germplasm Acquisition

Germplasm is collected to either fill gaps in existing collections or to protect forages at risk of disappearing. The objective in germplasm collection is to sample the most amount of diversity with a manageable amount of accessions. The strategy proposed by Marshall and Brown (1983) for forage species includes collecting seed from 50 to 100 individuals at each site, and sampling as many sites as possible to capture the range of environmental diversity. Sackville Hamilton and Chorlton (1995) outline strategies for collecting vegetative samples of forage grasses and legumes. Vegetative sampling requires additional effort to produce seed but may reduce effects of sampling time on preferential sampling of either early or late flowering genotypes. A possible compromise is to collect tillers with inflorescences of varied ripeness, immerse them in tap water and allow them to set seed in appropriate isolation. Collection sites and collecting details need to be thoroughly documented. Information such as geographic coordinates, ecological site description and improvement status (i.e. wild, landrace, cultivar) help plant breeders selecting germplasm since adaptation can be inferred from this data (Steiner and Greene 1996). Recent acquisition objectives for forages have focused on collecting wild relatives and landrace germplasm throughout Central Asia, in countries once part of the former Soviet Union (Street 2002, Greene et al. 2005). Efforts have also focused on collecting landraces and wild species from European countries (e.g. Chorlton et al. 2000, Annicchiarico 2006, Pederson et al. 1999). Francis (1999) provides a list of forage species that should be collected in the Mediterranean to broaden adaptation to specific edaphic conditions. Future acquisition efforts undoubtedly need to focus on forages that will expand current ecological niches to meet the challenges of global climate change.

4.2 Storing and Regenerating Forage Genetic Resources

Ex situ conservation of forages usually involves the storage of seed in gene banks. Standard temperatures for active collections are 0–4° C and for base collections −18°C, both at 3–7% seed moisture. Longevity in seed storage is dependant on seed increase conditions and initial germination going into storage. If seed is increased under optimal conditions and initial germination is >95%, longevity in storage for many forage species is forecasted to be 100 years or longer when stored at −18°C

(Sackville-Hamilton et al. 1998). Cryopreservation (−196°C in liquid nitrogen) of seed, meristem and callus is possible but is usually used for vegetatively propagated grasses (Cachită and Crăciun 1995, Reed et al. 2006). Germplasm storage conditions should be optimized to maximize longevity, since seed regeneration is the costliest activity of maintaining germplasm. Inevitably, newly collected seeds need to be increased for distribution, and have to be regenerated when seed quantity or viability drops below threshold limits. Careful consideration needs to be given to regeneration to minimize any genetic change during the process. This is especially true for forages, since they are largely out crossers and genetically heterogeneous. Genetic change can occur through genetic drift, selection, and contamination with alien genes (Sackville-Hamilton 1998). The effects of selection and contamination have the most impact (Sackville-Hamilton 1998, Van Treuren et al. 2006). Preferred and accepted standards for the different steps of regeneration were compiled by Sackville-Hamilton et al. (1998), and these standards are under continuous revision by the ECPGR working group on forages (Boller et al. 2005b, see also http://www.ecpgr.cgiar.org/Workgroups/forages/forages.htm). Choosing regeneration environments close to the original sampling environment minimizes selection pressure. However, costs of regeneration and accession sensitivity to environment also need to be considered (Hinton-Jones et al. 2007). Contamination by alien genes can be avoided in wind pollinated grasses by spatial isolation, and this can be improved by a tall barrier crop such as rye. Based on results of a recent international study (Marum et al. 2007), an isolation distance of 30 m with an efficient barrier crop is sufficient to limit alien contamination to 1% of pollination events. Contamination can be avoided in outcrossing legumes, which are mainly insect pollinated using isolation cages (Figure 3).

Differential seed production among maternal plants, as well as differential pollen production among paternal plants can decrease effective population size (N_e), causing population change through drift and selection (Johnson et al. 2002, Van

Fig. 3 Series of pollination cages for isolated seed regeneration of alfalfa accessions at Prosser (USA) (Photo S. Greene)

Treuren et al. 2006). This can be mitigated by harvesting equal amounts of seed or inflorescences from maternal plants (Johnson et al. 2004) or equalizing pollen contribution (Van Treuren et al. 2006). The additional labour costs of implementing these practices are substantial. The ECPGR forage working group recommends either keeping seed harvested from individual plants separate for samples going into the base or duplicate (i.e. regeneration) collection and making a bulk sample for distribution, or using at least 100 plants for regeneration when harvesting seed as bulk. Typically, harvesting 100 plants as a bulk limits overwhelming contribution to seed yield of some very big plants in the same way as making a balanced bulk of 30 plants harvested separately (Boller et al. 2009). Number of plants harvested, specific protocol used (for growing and harvesting accessions), and occurrence of any unusual environmental conditions should always be documented so curators and users can gauge for themselves the quality of the regeneration process.

4.3 Germplasm Evaluation

For PGR to be useful to plant breeders, accessions in *ex situ* collections need to be characterized and evaluated, and this information needs to be readily available to users of the collection. Generally, characterization focuses on traits that are simply inherited while evaluation focuses on traits that have quantitative inheritance. International Descriptor Lists have been published for forage grasses, forage legumes, annual medic sps., *Panicum miliaceum, P. sumatrense*, *Setaria italica*, *S. pumila* and *T. repens* (http://www.bioversityinternational.org/scientific_information/themes/germplasm_documentation/crop_descriptors/#c462). This provides a starting point for characterizing accessions in a standard format. Depending on resources, forage collections frequently have more data available. For example, over 1000 accessions of the USDA *Medicago sativa* collection have been evaluated for 13 diseases, 7 insects, 27 agronomic traits, 7 feed quality traits and 5 abiotic stress-tolerant traits (Bauchan and Greene 2002). This data can be queried and/or downloaded from GRIN (www.ars-grin.gov/npgs). In Australia, 20,997 annual medic accessions have been evaluated for 27 agronomic traits (Skinner et al. 1999). Qualitative traits can generally be collected during regeneration, but may not be of interest to plant breeders who are seeking disease, insect or abiotic stress resistance. Unfortunately the cost of germplasm evaluation is high, and frequently outside the scope of curators, who must focus on maintenance (Chapman 1989). This is particularly true for forages which generally exhibit large genotype by environment interactions due to their out crossing nature (Breese 1969). Another difficulty is that many forages are grown in a sward which adds additional cost in evaluating traits such as interplant competition, persistence and sward growth pattern, grazing tolerance and yield. Cost-effective protocols have been proposed including unreplicated designs, use of spaced plants and “micro-plots” (Annicchiarico 2004, Tyler et al. 1987, Rhodes 1987).

4.4 Major *Ex Situ* Collections

Ex situ genetic resource collections are unique in that they conserve important genetic diversity, but importantly, make it readily available to plant breeders and researchers. Table 3 lists PGR collections around the world with major holdings of temperate forage legumes and grasses with >1000 accessions. Contact information for requesting germplasm is also listed; in the case of decentralized collections like ECPGR, the easiest way to obtain seed is to contact the respective genebank curator. In the future, plant breeders will be able to do "one-stop-shopping", once a central internet portal is developed that links the world's genebanks (Global Crop Diversity Trust, 2009). Information on individual accessions is vital for helping plant breeders select germplasm with desirable characteristics. Most germplasm collections have passport and evaluation data, held either in local databases or online databases. The Germplasm Resources Information System (GRIN) database covers the United States collections (www.ars-grin.gov/cgi-bin/npgs/html/croplist.pl). Users can query online using a wide range of morphological, agronomic, disease- and insect-resistance descriptors to select accessions. The European Cooperative Programme for Plant Genetic Resource (ECPGR) has databases for the following forage grasses: *Agropyron*, *Agrostis*, *Arrhenatherum*, *Bromus*, *Dactylis*, *Festuca*, forage grasses (minor), *Lolium*, *Phalaris*, *Phleum*, *Poa*, *Trisetum* and forage legumes: forage legumes (minor), *Lathyrus*, *Lupinus*, *Medicago* (annual), *Medicago* (perennial) *Trifolium alexandrinum*, *T. resupinatum*, *T. pratense*, *T. repens*, *T. subterraneum*, *Vicia* and *Vigna* (www.ecpgr.cgiar.org/, click on Germplasm Databases). A database of genebanks can be found at www.bioversityinternational.org/scientific_information/information_sources/, and click on Germplasm Databases.

5 Strategies for Using PGR in Breeding

Historically, genetic resources have been instrumental in developing modern high-yielding forage varieties around the world. In New Zealand, for example, it has been estimated that germplasm introduced over the last 30–40 years contributes annually $1 billion to exports in grassland agriculture (Lancashire 2006). Although the value of PGR is well recognized, most plant breeders are hesitant to incorporate unadapted PGR into their programmes. Humphreys (2003) suggested the reasons are due to "the widening gap between improved/unimproved material, poor characterization of PGR, transfer of adverse traits, genetic disruption of background genotypes, genetic complexity of some traits and a slow rate of introgression". However, the benefits of broadening the genetic base of our cultivated fodder and amenity grasses, especially as we enter an era of uncertain environmental change, challenge us to build on traditional and new strategies to introgress PGR into breeding programmes. These strategies range from judicious selection of PGR, use of pre-breeding or population improvement schemes, to use of the latest molecular techniques to carry out "precision" breeding.

Table 3 *Ex situ* collections having major holdings of forage legumes and grasses

Country/international organization	Number of accessions	Description	Contact
Australia			
Australian Medicago Genetic Resource Centre, Adelaide, South Australia	45,640	Temperate pasture legumes of Mediterranean origin and Australian native forage species. Major genera: *Medicago* (mainly annual species), *Trifolium*, *Lotus*, *Astragalus*, *Hedysarum*, *Trigonella*, *Atriplex*, *Onobrychis*, *Melilotus*; >1500 amenity grasses	www.sardi.sa.gov.au/pastures/genetic_resources
Genetic Resource Centre for Temperate Pasture Legumes, South Perth, Western Australia	9,184	Pasture legumes collected from temperate regions around the world. Major genera: *Trifolium* (mainly annual species), *Ornithopus* and *Biserrula*	www.agric.wa.gov.au/content/PAST/PL/GENETIC_RESOURCE_INDEX.HTM
CGIAR			
International Center for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria	20,031	Forage and range species collected mainly between 45° and 30° latitude in Europe, Africa, Middle East and central Asia. Major genera: *Medicago* (annual species), *Trifolium, Astragalus, Onobrychis, Trigonella, Scorpiurus*	singer.cgiar.org/index.jsp

Table 3 (continued)

Country/international organization	Number of accessions	Description	Contact
Europe			
European Cooperative Programme for Plant Genetic Resources (ECPGR)	56,444	Forage legume and grass germplasm housed in over 35 institutes throughout Europe. Institute listings can be accessed at http://eurisco.ecpgr.org/. Major genera: *Trifolium*, *Festuca*, *Lolium*, *Dactylis*, *Medicago*. Strong representation of landraces and varieties developed in individual European countries, as well as local ecotypes.	www.ecpgr.cgiar.org/
United States			
USDA National Plant Germplasm System	33,142	Forage legume and grasses collected from temperate areas around the world. Range and wild species endemic to the United States. Major genera: *Medicago*, *Trifolium*, *Festuca*, *Elymus*, *Dactylis*, *Lolium*, *Eragrostis*, *Poa*, *Bromus*	www.ars-grin.gov/npgs/

5.1 Choice of PGR

The plant breeders' ideal PGR and the ideal PGR to benefit a breeding programme in the long term are generally opposed. Plant breeders are drawn to PGR for use in fodder crop or amenity grasses breeding programmes, that are genetically diverse, have promising characteristics (in view of the breeding objectives) and add genetic variability to the currently active breeding material. Furthermore, they should be reasonably adapted to the target environment. However, the greatest benefit, in terms of improving populations beyond current performance standards, and adding genetic variability would be from PGR that are most divergent from the germplasm used in the active breeding programme. However, these are in general, poorly adapted. Moreover, their characteristics are mostly unknown, at least in their response to the target environment. At the other end of the scale, new varieties from an outside breeding programme which have proven their value in recent official variety trials would be ideal candidates in the search for well-documented promising characteristics. However, their potential to add genetic diversity to the pre-existing current breeding material is doubtful. On the one hand, new varieties from a successful breeding programme are likely to be closely related to older varieties of the same programme which had already been used previously. On the other hand, older varieties of one's own breeding programme may have been used in the development of outside breeding programmes. Furthermore, exchange of breeding materials due to changing alliances among breeding companies has probably decreased genetic diversity between the breeding pools used by different breeders.

Selection from local and introduced ecotypes (especially if germplasm originates from a different gene pool than the breeding pool) can be expected to provide a good compromise between the requirement of adding genetic diversity and that of offering promising characteristics along with good adaptation to the target environment. If environmental data of their origin are known, their adaptation can be matched with the target environment of the breeding programme. Increasingly, germplasm collections are improving on the quality of passport data, especially more precise information on latitude and longitude. Text descriptions of older collection site locations are being converted into geographic map coordinates and new collection sites are being documented with GPS. This allows plant breeders to more easily select germplasm adapted to their target environment. Once GIS-based applications are coupled to genebank collection databases, selection of adapted germplasm will be further facilitated (Greene et al. 2007). Interesting characteristics are usually less well known than with varieties but may be derived from knowledge of environmental and management factors of the site the ecotype originate from.

In situations where adapted ecotypes do not provide the needed variation, plant breeders must turn to unadapted material, and in some cases (especially in grasses), utilize germplasm of different species and even genera. Plant breeders rarely have the resources to evaluate entire collections for traits of interest. The choice of PGR can be simplified by carrying out an initial screening of a core collection, if available. The core collection concept was proposed by Frankel (1984) who suggested

that a subset of accessions from a germplasm collection could be identified that represented the majority of genetic variation in a collection. A core collection that contained 5–10% of the accessions should retain over 75% of the variation of the entire collection (Brown 1989). There are numerous ways to develop core collections but the general strategy is to hierarchically stratify the collection into classes of accessions that share common characters. Characters can include ecologic or geographic origin, phenotypic characters, molecular marker data or a combination. Once the collection is classified, accessions are sampled from each class, using a number of different sampling strategies (Van Hintum 1999). Theoretically, after evaluating a core collection and identifying useful accessions, plant breeders can go back to the whole collection and screen those accessions in the same class(es) as those identified in the core subset. Core collections have been developed for most of the major forage germplasm collections. The USDA germplasm collections have core collections of red clover (Kouame and Quesenberry 1993), white clover, birdsfoot trefoil (Steiner et al. 2001), annual medic (Diwan et al. 1994), alfalfa (Basigalup et al. 1995) and *Poa pratensis* (Johnson et al. 1999). A core collection has been developed for the Australian Annual Medic Germplasm collection (Skinner et al. 1999) and *Medicago truncatula* collection (Ellwood 2006). A core collection for *M. truncatula* has also been developed for the French collection (Ronfort et al. 2006). A core collection is also being developed for the European collection of *L. perenne* (Maggioni et al. 1998).

5.2 Pre-breeding Strategies

Although careful choice of PGR can help overcome some of the challenges of using PGR, to truly capitalize on the presence of unique alleles outside of the breeding gene pool, plant breeders need to adopt strategies that will allow the introgression of unadapted germplasm into the breeding programme as efficiently as possible. Traditionally, the approach has been hybridization and backcrossing to the elite germplasm. Test cross evaluation allows breeders to further evaluate unadapted germplasm, especially for quantitiative characters such as yield, as well as start of the pre-breeding process. Crosses are made between unadapted germplasm and elite genotypes and the progeny evaluated for the trait of interest (for example, Bhandari et al. 2007, Maureira et al. 2004). This can uncover potential traits that might be masked in unadapted germplasm. Also, estimates of GCA and SCA can provide insights into additive and non additive gene action and suggest which unadapted germplasm might be most beneficial to incorporate into a breeding programme. Test cross progeny can be bulked to form composite populations or gene pools. Williams et al. (2007) developed several white clover varieties in New Zealand using this technique. The formation of regional gene pools was proposed to broaden the genetic base of alfalfa in the United States (Barnes et al. 1977). Regional genepools of perennial ryegrass were established in France (Charmet and Balfourier 1995). A further pre-breeding approach was undertaken in Germany where 800 Polish genebank

accessions were evaluated and genepools created according to the same time of flowering (Paul 1989).

Interspecific, and even intergeneric hybridization, has been successfully employed, and varieties released in several grasses, most notably in *Lolium* and *Festuca* species (Humphreys 2003, Humphreys et al. 2006). Interspecific hybridization has also been used to transfer traits such as rhizome production, seed production and persistence in *Trifolium* (Abberton 2007), and disease resistance and pod coiling in *Medicago* (Armour et al. 2008). Although no varieties have been yet commercialized, this can be expected in the near future (Williams and Hussain 2008).

Advances in genomics should make introgression of unadapted germplasm much more efficient. Germplasm collections may be "mined" for desirable genes to introgress into elite lines (Tanksley and McCouch 1997). The basic strategy involves the development of genetic linkage maps. The location of targeted genes and quantitative trait loci (QTL) can then be identified. Molecular markers that are closely associated with the gene of interest will then allow for marker-assisted selection (MAS). In forages, rapid progress is being made to develop these genomic tools for use in breeding programmes (see Chapter 4). However, before these genomic tools will truly allow plant breeders to more effectively and efficiently utilize PGR to improve production and broaden the genetic base of cultivated fodder and amenity grasses, functional markers must replace the anonymous markers which are still dominating research into localizing QTLs (Kölliker et al. 2009).

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Breeding Methods in Cross-Pollinated Species

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1 Introduction

Plant breeding is the genetic improvement of plants and is considered to be both a science and an art. The latter is governed by the breeder's eye and his intuition and creativity. The scientific basis of plant breeding methods was established in the first half of the last century after the rediscovery of Mendel's laws. Population and quantitative genetics made essential contributions towards the development and understanding of breeding methods. These methods are not recipes, but they provide a framework of guidelines through the breeding process towards the creation of an improved variety. Breeding history in forage crops is less than 100 years old, and the breeding of amenity grasses has lasted for less than 50 years. Selection was facilitated by intensified evaluation in the last three decades due to the invention of plot harvesters, data loggers, electronic systems, computing technology and more recently NIRS-online systems.

One should bear in mind, however, that the breeding methods applied in forage crops and amenity grasses mostly evolved from the breeding of field crops, especially maize. In most plant breeding textbooks breeding methods are described and explained according to the mode of reproduction of the particular crop. A breeding method comprises all the necessary breeding steps from the choice of source materials up to the final selection among candidate varieties. The creation of new varieties meeting the requirements of the end-users is the ultimate goal of every breeding programme. Besides the choice of an efficient breeding method, the designation of a promising complex of objectives and the selection of appropriate source materials are of fundamental importance. The breeding methods are specific, not so much to crops, but rather to modes of reproduction and to types of varieties to be bred. In this chapter we will follow the systematics of breeding elaborated by Schnell (1982), who classified breeding methods according to the propagational type of the resulting varieties into *four breeding categories*: *line, population, hybrid and clone breeding.*

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1.1 Reproduction and Mating Systems

Three main types of reproductive systems are described in the literature (Evans 1964). Fryxell (1957) proposed the reproductive triangle as presented in Figure 1. The four classes of varieties can be linked to the modes of reproduction.

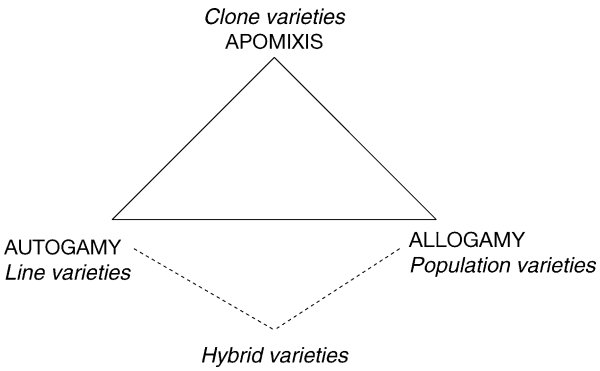

Fig. 1 The reproduction triangle with the modes of reproduction (in capital letters) and the four breeding categories and resulting types of varieties

Among the vast range of temperate grasses, all three types of sexual and asexual modes of reproduction are present. The reproductive triangle as proposed by Fryxell (1957) also implies that there are no strict barriers between reproduction systems, but a gradual transition from one mode to the neighbouring one. In the genus *Poa* all three modes are present, and within *Poa pratensis* populations' asexual propagation via apomixis as well as cross-fertilization can occur for particular genotypes. Thus, from strictly apomictic plants *clonal varieties* can be selected, while *population varieties* will be bred from the latter types.

Hybrid breeding, as shown in Figure 1, involves both autogamy and allogamy and could be called a man-made breeding system. Besides the special case of apomixis in Poa, which is treated in Chapter 15, and line breeding in some autogamous annual forage legumes (Chapter 20), all other species covered in this volume are cross-pollinating, and thus the application of population breeding is most prominent.

The four types of varieties can be grouped (Figure 2) according to their genetic and phenotypic variability (homogeneity or heterogeneity) and their genetic constitution (homozygous or heterozygous). Maximum heterozygosity can be achieved in single-cross hybrids. To fulfil DUS requirements, population varieties need a certain level of phenotypic homogeneity (uniformity), but at the same time they should have as high a level of heterozygosity as possible, due to the relationship with the level of performance.

With the single exception of annual ryegrass, all forage and amenity grass species are perennials and can be maintained vegetatively for many years under sward conditions, while under spaced plant conditions individual plants or clones (vegetative progeny of an individual) have to be sub-cloned after a few years, depending on

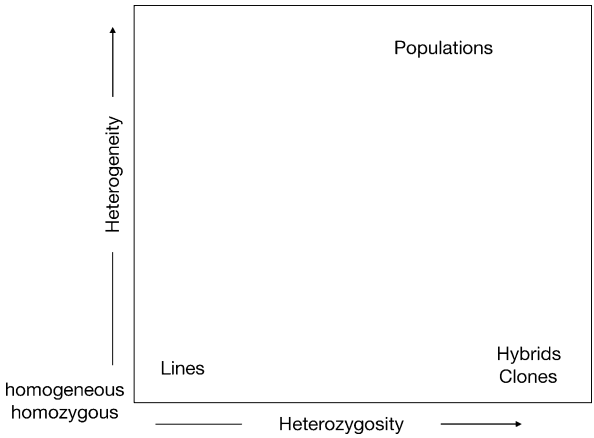

Fig. 2 Genetic structure of the four basic types of varieties (Becker 1993)

the species. The perennials have vernalization requirements (short days and low temperature) for floral induction. During anthesis, anthers release clouds of pollen which are windblown onto the stigmas to effect pollination. The ryegrasses have a two-locus gametophytic incompatibility system (Cornish et al. 1979) in common with rye (*Secale cereale* L., Lundquist 1956). Under normal conditions this system prevents self-pollination. Seed set after enforced selfing is largely genotype dependent (Posselt 1982), but can be improved by thermal treatment during anthesis (Wilkins and Thorogood 1992) to overcome self-incompatibility in perennial ryegrass. Highly self-fertile inbred lines have been developed (Utz and Oettler 1978). Using partially inbred lines, self-incompatibility (SI) can be used to construct SI-hybrids (Posselt 1993).

Alfalfa, red and white clover are also perennials, but of shorter individual lifetime than most grasses. All of them have an insect-aided cross-pollination system. The leguminous forages too have a gametophytic incompatibility system which is under control of a single locus. Selfing in these species is possible; however, tripping by hand is essential and in the absence of genetically controlled self-compatibility, seed set is rare (Poehlman 1979). Vegetative propagation is easy to achieve through stolon cuttings in white clover and feasible, though less prolific, through stem cuttings or crown separation in alfalfa and red clover.

Polyploidy is widespread among forage crops and amenity grasses. Comparatively few species are true diploids. Many grasses and forage legumes are autopolyploids or allopolyploids. In the latter, disomic inheritance is assumed. Autotetraploidy can be easily induced by colchicine treatment in naturally diploid ($2n=14$) species such as Italian, perennial and hybrid ryegrass, meadow fescue and red clover. The genetic theory of autotetraploids is rather complex. The most relevant topics in the breeding of tetraploids will be dealt with in a separate section.

Mating designs are important features in selection theory and have been developed to (i) control pollination and (ii) create particular types of progenies (see Section 2.2). In allogamous species large breeding populations will be multiplied by

open pollination which gives the name for *open-pollinated varieties* (OPV). Seeds will be harvested as a bulk, which is also the case in *mass selection*. In the *polycross* replicated clonal parts of all genotypes produce a huge pollen cloud which ensures high recombination. Furthermore, enough seeds for plot trials can be harvested. In the *top cross* layout candidate genotypes will be pollinated by a tester. Whenever open pollination is applied, isolation requirements have to be considered. Controlled matings take place in *paircrosses* where two plants are bagged together. Mutual pollination is assumed and in general no emasculation is performed. The most restricted mating is *selfing*. In cases where enough seed for replicated plot trials are wanted paircrosses as well as selfings are performed with clonal parts in seed islands of a field of rye or triticale.

2 Breeding Population Varieties

According to Schnell (1982), two types of population varieties can be distinguished: Open-pollinated varieties (OPVs), which are the result of population improvement through recurrent selection, and synthetic varieties. "A commercial synthetic variety is an advanced generation of a population initiated by crosses among a restricted number of [GCA] selected parents and multiplied by a number of random outcrossing in isolation" (Wright 1981). Both OPVs and synthetic varieties constitute panmictic populations, since they are produced by random fertilization, at least in the advanced generations of seed production (Schnell 1982).

To structure the various breeding methods, Schnell (1982) suggested a partition into three breeding phases:

1. Procuring initial variation
2. Forming varietal parents
3. Testing experimental varieties

In brief, in the *first phase*, the base population is created. If non-adapted materials shall be used pre-breeding may be necessary before phase 1. The *second phase* comprises selection of the best individuals as the immediate parents of the first generation used to construct experimental varieties, or to create an improved breeding population. Since the likelihood of success after one step of selection is rather poor, population improvement through recurrent selection is typical in population breeding and is inevitable for future breeding progress. In the *third phase* experimental varieties are constructed and tested. In this phase, procedures differ between schemes of OPV and synthetic breeding. These two approaches are discussed in Section 2.3.

2.1 Creation of the Base Population

The most important requirement for base materials that will be used for establishing the base population is a high frequency of positive alleles for the traits of interest.

This can be achieved by synthesizing base materials that are as unrelated as possible (Geiger 1982).

Principle source materials are

- wild relatives;
- ecotypes (from own collections or genebanks);
- landraces (Hofsorten, farm varieties);
- improved breeding materials (populations, families, clones, inbreds);
- released varieties (OPVs, synthetics).

Wild relatives are mostly not competitive because of the lack of adaptation. However, they can provide genes for specific plant characteristics such as resistance to disease and drought. Low-performing materials like wild relatives or unadapted ecotypes should be used as gene donors only if more advanced materials are not available for that purpose. Introgression of such genes should be carried out by repeated backcrosses to high-performing breeding materials before establishing the base population (Geiger 1982).

Within the groups' ecotypes, landraces and released varieties, adapted and non-adapted germplasm can be distinguished. In contrast to arable crops, there are rarely landraces as a bridge between wild and cultivated forms within perennials. According to the rather short breeding history of most of the species concerned, many breeders and researchers regard these species as still being wild, since a real domestication process like in arable crops did not take place. This topic has been discussed in more detail by Casler et al. (1996). In many cases no clear distinction between ecotypes and bred varieties, neither by phenotypic characters nor by means of molecular markers, has been found. Thus, it seems to be more appropriate for the breeder to distinguish only between adapted and non-adapted materials.

Diversity among and within populations can be measured on the phenotypic (morphological characters) and on the genotypic (molecular markers) level. Relatedness among source materials can thus be quantified. Unrelated materials are considered to be more diverse judged from gene frequencies than related ones. The magnitude of heterosis in the hybrid from a cross between two populations is a direct indication of the level of divergence among populations, and is used for the establishment of divergent gene pools in hybrid breeding programmes (see Section 3). In population breeding greater genetic variability within such a newly formed population can be expected. In perennial ryegrass a close relationship between geographic distance and hybrid performance ($r = 0.64$) was found (Figure 3). In maize, Moll et al. (1965) demonstrated the relationship between geographic distance and genetic diversity.

In cocksfoot, crosses among European and American varieties showed hybrid effects directly proportional to the putative genetic distance between the parents (Christie 1970, Christie and Krakar 1980). During the last decade molecular markers were applied in several crop species to investigate diversity patterns of the respective gene pool.

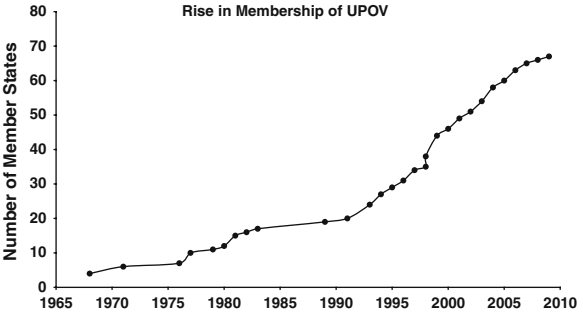

Fig. 3 Geographic distance and hybrid performance of diallel crosses among perennial ryegrass populations

The geographic origin of ecotypes is mostly known to the breeder. For released varieties, the situation is rather complex, since breeders have intermated whatever materials they had at their disposition, no simple grouping of varieties is anymore possible. In a molecular marker study with German ecotypes and varieties of perennial ryegrass it was shown that only 2% of the total genetic variation could be attributed to variation between these two groups of material (Bolaric et al. 2005). Similar results were reported by Peter-Schmid et al. (2008) for Italian ryegrass, but somewhat greater proportions for meadow fescue.

When choosing the sources of germplasm the breeder should have already clearly defined his breeding objectives. Instead of directly intermating unknown materials, it is worthwhile to evaluate the source materials according to the traits of interest. Furthermore, a preliminary grouping of the materials according to time of flowering and relatedness facilitates the establishment of the base population.

Referring to maize breeding Hallauer and Miranda (1981) stated the following:

> Choice of germplasm is a critical decision that requires considerable thought. Hasty decisions either to eliminate or to decrease number of growing seasons required may in the long run increase the number of growing seasons required to develop usable materials. In many instances the selected germplasm will be the basis of the breeding program for the lifetime of the breeder. Choice of germplasm will determine maximum potential improvement that can be attained via breeding; the breeding system used will determine how much of that maximum potential can be realized.

The number of genotypes selected for the creation of the base population and the mode of intermating vary according to the objectives and technical facilities available (see Table 1). Furthermore, the breeder has to decide whether to establish one or several base populations, and if these will be used in short-term or long-term selection programmes. Especially in research studies, mostly “closed” populations are used for the purpose of comparing response to selection. In practical breeding programmes, the option of “open” populations is more promising. The upgrading of the breeding population can be done for the following reasons: (i) increase of the already reduced genetic variance, (ii) avoidance of inbreeding through the introduction of unrelated material and (iii) introgression of new genes affecting newly defined traits of interest. Depending on the purpose of upgrading, a back-up of the original base population which underwent some type of relaxed selection could

Table 1 Examples of base population construction

Origin	Single source	Multi source	
Genetic base	Broad	Narrow	Very broad
Genetic material	Regional ecotypes	Distinct genotypes	25 varieties
No. of parents	100	8	100
Population size	1,440	3,500	13,000
Reference	Charmet and Debote (1995)	Wilkins (1985)	Johnston and McAneney (1994)

already fulfil most of the requirements made. Otherwise, narrow breeding or backcross populations, superior families or other so-called elite breeding material could be useful in broadening the genetic base of the respective base population. After creation of the base population it may be worthwhile estimating the overall performance of the population. In general, populations with a large phenotypic variation have lower means than those with a narrow base.

Knowledge of performance per se enables a comparison with the best current varieties. If one assumes that a base population yields only 10% less than the best variety, and that the gain from selection (per year) is 1.5% in the base population compared to a general gain in advanced breeding populations of 0.5%, then it will take at least 10 years until the new population becomes agronomically competitive. In this context, Baenziger et al. (2006) stated, "A key issue in using hybridization to create new variation is selection of the parents. Despite the obvious importance of this issue, much more research has been done on methods of selection in breeding populations than on selection of parents to create these populations."

2.1.1 Selected Topics from Population Genetics

A population can be defined as a community of individuals which share a common gene pool (Allard 1960). In a large random-mating population with no selection, mutation or migration, the gene frequencies and the genotype frequencies are constant from generation to generation. Such a population is said to be in *Hardy–Weinberg equilibrium* (HWE) (Falconer 1989). If a representative sample of plants (large number) is drawn from such a population and intermated under panmictic conditions (equal chance of all male and female gametes to match), the resulting next generation will contain the same allelic and genotypic frequencies as the previous one. However, genes are regrouped into new genetic combinations. Thus, a particular genotype is not maintained into the next generation. While theoretically following the law of HWE with respect to any one locus, there are factors upsetting HWE: non-random mating, mutation, migration, random drift and natural selection, as explained below.

Random mating is probably the most important assumption with respect to HWE and is often violated in practical breeding, mostly because of low numbers of parental genotypes and of poor intercrossing designs. A population not in

HWE is not suited as a reference population in quantitative genetic studies, since most of the assumptions are related to HWE. Furthermore, the mean and the variance of that population may be different from those of its next generation. The implications for practical breeding are obvious, and selection starting from poorly equilibrated base populations is like gambling. During creation of the base population it is therefore advisable to make a second round of intermating to reach HWE. Even further propagation is advisable, since linkage disequilibrium will be reduced and formerly linked loci will freely segregate and new recombinants may occur.

Mutations occur at low frequency, but are mostly recessive and will be masked in the heterozygous state. Gross mutants like chlorophyll deficiencies will be easily recognized. However, many deleterious recessive alleles will only become visible during inbreeding. The occurrence of lethal and semi-lethal genes in panmictic populations is called the genetic load. *Migration* takes place from volunteer plants, e.g. from impurities acquired during seed cleaning, or when the population is not properly isolated during pollination and foreign pollen can migrate. *Random drift* occurs when insufficient numbers of individuals are used to propagate the population. Gene frequencies will vary from generation to generation and not reach HWE. Thus, genotypic frequencies will not be stable across generations, which is an important feature in maintaining an OPV. Furthermore, in small populations alleles can get lost or become fixed. Selection in small populations result in intermating of related genotypes and inbreeding may therefore occur. The magnitude of inbreeding, expressed as F (coefficient of inbreeding), depends on N_e, the effective population size. With increasing F the additive variance is reduced in a proportional manner (Gallais 1990). *Natural selection* (also called shift) favours frequency of alleles conferring resistance to the stress imposing a selective force. A typical example is the small population consisting of the surviving populations in the breeding nursery after severe winters, when only frost-tolerant genotypes will have survived. It is obvious that the breeder would like to immediately take profit from this selection event and to create a variety out of this outstanding material. However, there are some points to consider: (i) genes positively affecting the particular trait may already be fixed in the survivor population, (ii) genes for other important agronomic traits might have been lost and (iii) the probability that the survivors are closely related is high. Thus, apart from the particular trait, overall performance will not be sufficient, inbreeding will occur during multiplication and last but not least, if the genes are fixed there will be no genetic variation and thus no response to further selection. It will therefore be more appropriate to use this population as one of the sources for the creation of a new base population.

2.1.2 Broadening the Gene Pool

During evaluation of the source materials it may happen that for particular traits little or no variation is observed in the gene pool. There are several approaches to

solve this problem: gene transfer by backcrossing if the trait of interest is available in other gene pools, induced mutation and introgression through interspecific and intergeneric hybridization.

Backcrossing is a valuable breeding technique in transferring a single desired character to an otherwise superior material (the recurrent parent) without inducing other changes. Success from this approach depends largely on (i) the availability of a good recurrent parent, (ii) the identification of the transferred character in segregating populations and (iii) undesirable characters not being closely linked with the desirable character to be transferred from the donor (non-recurrent) parent. This technique has been very useful in transferring simply inherited traits, especially major genes for resistance (Hanson 1972). To increase seed retention, backcrossing was applied in cocksfoot (Falcinelli 1991). For quantitative traits the sample size of the recurrent parent should be about as large as N_e in population improvement (Geiger 1982). This makes backcrossing in allogamous species less attractive compared to autogamous or clonally propagated species.

Mutation breeding can be sought successfully for specific characteristics where the range of natural variation is limited. Although most induced mutations are of no practical value, available data show that beneficial mutants can be isolated and used in crop improvement (Hanson 1972). Mutational treatment, whether with chemical mutagens or ionizing radiation, is often applied in already improved materials or released varieties. Some successful varieties such as red clover "Larus" (Boller et al. 2001) or meadow foxtail "Alko" (Simon 1994) have been developed in this way. In the latter species seed retention was substantially improved.

Introgression aims at the incorporation of desirable traits through controlled intergeneric or interspecific hybridization followed by backcrossing with the recurrent parent. Hybridization between plant species has occurred in nature, especially among the grasses, and is an important mechanism of plant evolution (Casler et al. 1996). In many cases hybrids between species are sterile and chromosome doubling through colchicine treatment is necessary. Breeding efforts in the cool season grasses have focused on the ryegrass–fescue complex (Thomas and Humphreys 1991), mostly with the objective of introgression of drought tolerance from the fescues into the ryegrasses (Humphreys and Thomas 1993). The "stay green" gene has successfully been transferred from *F. pratensis* into *L. perenne* amenity types (Thorogood 1996). Crown rust resistance has been transferred from *F. pratensis* to *L. multiflorum* (Oertel and Matzk 1999).

The production of hybrids between more distantly related grasses, so-called wide crosses, has been reported by Matzk et al. (1997). These authors obtained allopolyploid hybrids between important forage grasses (*L. multiflorum* × *Dactilys glomerata*; *F. pratensis* × *D. glomerata*; and *F. rubra* × *L. perenne*). However, the material has not reached advanced breeding status. Hybridization between closely related species or genera was used to create new species from which many successful cultivars have been developed: hybrid ryegrass from crosses between perennial × Italian ryegrass (see Chapter 10), and Festulolium from crosses between *Lolium* spp. × *Festuca* spp. (see Chapter 12).

2.2 Population Improvement – Recurrent Selection

"The choice of any one method of selection depends on the breeder, stage of the breeding program, stage of germplasm development, stage of knowledge of the populations and objectives of the breeding program" (Hallauer and Miranda 1981). Recurrent selection (RS) is defined as any breeding system designed to increase the frequency of desired alleles for particular quantitatively inherited characters by repeated cycles of selection (Sleper and Poehlman 2006). *Selection cycles may be repeated as long as superior genotypes are being generated*. The improved population can be used as a variety per se (OPV) or as a source of superior genetic units that can be used as parents of a synthetic. In general intrapopulation improvement is applied in a single breeding population. In special cases of synthetic breeding, population improvement with several divergent populations may be carried out.

Individuals in a population can be evaluated on the basis of their phenotype or on the basis of the performance of their progeny. *Phenotypic selection* can be based on individual plants or their vegetatively propagated progenies (clones) in single (clonal rows) or replicated plots. The evaluation can be made by visual inspection (scoring) or by measuring the character of interest (Fehr 1987). In breeding research, data from all entries will be collected, whereas in practical breeding unwanted plants are often eliminated in each round of inspection to reduce the amount of future scoring.

Genotypic selection of an individual plant is based on the performance of its progeny. The terms half-sib (HS) and full-sib (FS) refer to the genetic relationship among individuals within a family. A series of half-sib families (HSF) are formed in PX and TX schemes by crossing a series of individuals to a common population of pollen parents. Full-sib families (FSF) are created by crossing pairs of plants. Half-sib families are related with each other because they share a common parent, but full-sib families have no parents in common (Fehr 1987). Selfed progenies derive from selfing individual plants or clones or from intra-family mating.

The method of selection is classified by the type of *selection unit*, which allows quantitative genetic interpretation. In Figure 4 a generalized scheme is given and the terms are described as

- *selection units*: plants, clones or progenies
- *test units*: plants, clones or progenies evaluated to provide the data used as the basis for selection
- *recombination units:* plant, clone or progeny that is finally recombined to establish the improved population.

In mass selection the selected plants will be directly used for recombination, i.e. selection, test and recombination units are the same. With progeny-testing methods alternative pathways are possible. If the selection units can be preserved vegetatively, either the progeny or their parents (PU – parental unit) can be used for recombination. Thus, in some selection schemes such as that in Figure 4 only

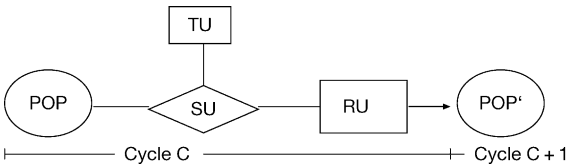

Fig. 4 Generalized scheme of recurrent selection (Schipprack 1993) (POP: population, SU: selection units, TU: test units, RU: recombination units)

the information from the progenies (test units) is used to select the parents for recombination.

2.2.1 Phenotypic Selection

Mass selection was one of the earliest selection procedures to be used in cross-pollinating species. It is described by Sleper and Poehlman (2006) as a selection procedure in which individual plants are chosen visually for their desirable traits and seed harvested from selected plants is bulked to grow the following generation without any form of progeny evaluation. Continuous mass selection for a specific character with high heritability that can be evaluated visually, such as time of flowering or disease resistance, will shift the gene frequency of the character in the direction of selection. The shift towards more adapted genotypes may be accelerated by selection under environmental stresses to which the breeding population is subjected (Sleper and Poehlman 2006). The advantages of mass selection are high selection intensities and the shortest possible breeding cycle.

The limitations in mass selection are the environmental effects and the genotype × environment interaction which mask the genotypic value. To reduce environmental variation, Gardner (1961) suggested gridding of the spaced plant nursery, where, e.g. the best 4 plants from each grid of 40 plants are selected. Another weakness of mass selection is the lack of control over the pollen source and the genes contributed by the male gametes. The breeding nursery must be isolated from other pollen sources of the same species to avoid uncontrolled gene flow. Selected plants are open pollinated and random mating depends largely upon the total number of plants and the distances among them. When using the gridding suggestion of Gardner (1961), with an interplanting distance of 50 × 50 cm, a grid of 40 plants covers 10 m^2 from which 4 plants will participate in open pollination. Having started with 8,000 plants (200 grids), the selected 800 plants will be rather evenly distributed across 2,000 m^2. Nevertheless, selected plants will be preferentially pollinated by their closest neighbours. If selected plants are nested, random mating is even more questionable.

In forages and amenity grasses most traits are evaluated during vegetative development and selection decisions can be made before flowering and unwanted plants can be eliminated in time (Figure 5). Since only selected individuals are recombined, parental control is on both sexes (c=1). For post-flowering traits like seed yield characters, intermating of the selected plants has to be postponed to the following year to be as efficient as selection before flowering. With selection after flowering only the female parent is under control (c=0.5).

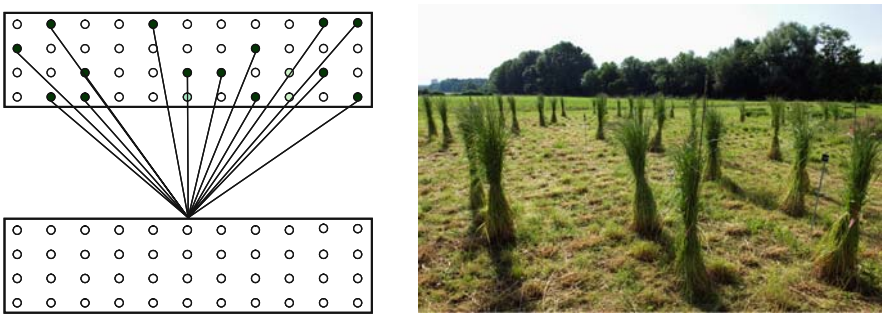

Fig. 5 Mass selection with pollination control before flowering (Photo G. Brändle)

Other attempts to improve pollination control are (i) vegetative propagules from the selected plants may be transplanted into an isolated crossing block using a polycross design or (ii) culms from all selected plants bearing inflorescences can be removed just prior to anthesis and placed in water or nutrient solution in an indoor isolation. During pollen shedding the culms should be shaken to enhance pollen dispersal and random pollination. In the RRPS (recurrent restricted phenotypic selection) system designed by Burton (1974), gridding or stratified planting and the detached culm technique are essential prerequisites.

Before seed harvest the breeder has to decide how to establish the representative bulk from which plantlets are grown for the next cycle of selection. Separate harvesting of each plant or detached culm enables the establishment of a bulk of seed with equal amounts from each parent genotype to avoid uncontrolled selection among the parents.

An alternative to mass selection is *maternal line selection* which is applied in red clover breeding in Europe, in alfalfa breeding in Canada (Poehlman 1979) and in timothy breeding in Japan (see Chapter 14). In brief, seed from each seed parent plant is kept separately and the progeny is planted in unreplicated rows (i.e. ear-to-row in maize breeding). Due to open pollination among the parents, only the female side is known and the progenies are half-sib families. Selection can be practised as with the grid system, among and within families. Intermating of the selected plants follows one of the procedures already described. In this system intermating of close relatives can be avoided by selecting only one individual per family.

Clone selection can be used to evaluate clones instead of individual plants. In practical breeding mostly clonal rows are planted instead of randomized experimental designs as in research. With clonal rows several cutting treatments (none, infrequent, frequent) can be applied, and thus several vegetative and generative traits can be compared. Breeders must be aware that selection in a single environment bears the risk of selection for specific adaptation. Furthermore, selection pressure of diseases varies from year to year and is seldom evenly distributed across the nursery. Artificial inoculation helps to overcome these drawbacks, improves heritability of resistance traits and secures selection decisions. Alternatively, clonal parts of

all members of the population under improvement can be grown in highly selective environments. A further approach to minimize environmental effects is the so-called shuttle breeding, where the evaluation of the whole breeding population alternates between two divergent test locations.

If the replicated clones are a random sample, phenotypic and genotypic variance as well as heritability for the traits of interest can be estimated and will describe the reference population (Frandsen et al. 1978). Using phenotypic selection, a breeder selects on the basis of the phenotype of an organism, but really selects a genotype (Frey 1983).

Mass selection should be successful if

- gene action for the selected trait is primarily additive;
- heritability of the trait is high;
- traits selected on individual spaced plants and traits to be improved in a dense sward are genotypically correlated;
- the effective population size (N_e) is large enough to sufficiently limit inbreeding depression (see Table 2).

In practical breeding, often about 10% of the individuals are selected, corresponding with a selection intensity of $i = 1.755$. However, simulation experiments (Vencovsky and Godoi 1976) revealed that for short-term response a low selection pressure 500/5,000 was inefficient and these authors advised increasing selection pressure to 1% ($i = 2.66$) or even 0.5% ($i = 2.89$). This high selection intensity is one of the advantages of recurrent mass selection. The inbreeding coefficient F depends on the effective population size N_e and the level of inbreeding in the previous generation and can be calculated according to Falconer (1989):

$$F_t = \frac{1}{2N_e} + \left(1 - \frac{1}{2N_e}\right) F_{t-1} \tag{3.1}$$

From Table 2 it is obvious that doubling of N_e approximately halves the inbreeding coefficient. The effective population size (N_e) plays a decisive role in the progress of population improvement since it acts in opposite ways on the selection intensity on the one hand and on inbreeding and random fixation on the

Table 2 Expected levels of inbreeding after t cycles of panmictic recombination within a population of N_e individuals with $F = 0$ in the base population, according to formula (3.1)

	Effective population size N_e				
Cycle T	10	20	30	40	50
1	0.0500	0.0250	0.0167	0.0125	0.0100
2	0.0975	0.0494	0.0331	0.0248	0.0195
3	0.1426	0.0731	0.0493	0.0370	0.0285
4	0.1855	0.0963	0.0651	0.0491	0.0371
5	0.2262	0.1131	0.0806	0.0610	0.0467

other hand. The fewer genotypes the breeder selects for recombination, the greater will be the superiority of the selected fraction, but the more the population will suffer from inbreeding depression, the faster will its genetic variability be exhausted and the lower will be the limits of selection. It is usually less disadvantageous to work with a large N_e, even at the expense of a lower selection intensity, than to go for a too small N_e. Conditions demanding large N_e are small genetic variance, high sensitivity to inbreeding and large number of tested candidates (Geiger 1982). The performance of the improved population should be measured in replicated plot trials in comparison with the best varieties on the market.

2.2.2 Genotypic Selection

In a narrow-based population, or after several cycles of phenotypic selection, visual differentiation among individual plants becomes rather erratic and unreliable for traits with low heritability (h^2<0.20). The purpose of progeny testing therefore is to estimate genetic variance by separating environmental from the phenotypic variance by means of replicated trials. To avoid genetic sampling effects, the number of plants per replicate should be chosen according to the type of family. For half-sib (HS) families 15–20 plants are adequate, whereas for full-sib (FS) families somewhat lower numbers might be used. Comparison *among families* is based on entry means across replicates. Plot trials to test the families are named *progeny test* (HSPT or FSPT) in the literature (Sleper and Poehlman 2006, Casler and Brummer 2008).

In spaced plant trials selection is often practised as *among and within family* (AWF) selection, selecting the "best" individuals of the "best" families (Nguyen and Sleper 1983, Vogel and Petersen 1993). Aastveit and Aastveit (1990) presented experimental data from a selection experiment with meadow fescue. They also calculated response to selection for several combinations of AWF selection. Casler and Brummer (2008) recently presented expected genetic gains of AWF selection for HS and FS families and concluded that AWF selection was superior over mass selection. However, these types of spaced plant trials do not allow for a separation between the genotypic and environmental variation within families, unless each member of each family is clonally replicated (Aastveit and Aastveit 1990).

Half-Sib Families According to selection theory the covariance among non-inbred HSFs is $^1/_4$ of the additive variance (σ^2_A) and equals the general combining ability (GCA). GCA is defined as the average performance of a genotype in a series of crosses, and is measured as the deviation of its progeny from the mean of those crosses. GCA values are especially useful in the prediction of synthetics and hybrids.

Half-sib families can be obtained in several ways. Allard (1960) described four mating systems to produce HS progenies: Open pollination (OP), polycross (PX), topcross (TX) and diallel matings. The latter is rarely used in practical breeding because only small numbers of parents can be investigated. OP progenies derive from individual "female" plants and an unknown pollen cloud (see maternal line

Fig. 6 HS family selection scheme and a topcross nursery (tester are drilled rows) (Photo U.K. Posselt)

selection), whereas in PX and TX progenies, a homogeneous, common pollen cloud is assumed (see Fig. 6).

The *polycross test* (PX) was developed independently in Denmark (grasses), the USA (alfalfa) and the Netherlands (rye) by Frandsen (1940), Tysdal et al. (1942) and Wellensiek (1947), respectively. The latter named his method mass test cross, while the term *polycross* was suggested by Tysdal et al. (1942). A prerequisite is the possibility for vegetative propagation (cloning) of a crop. The clonal propagules are interplanted in such a way that each of them is neighboured by clonal plants derived from different genotypes. To assure random mating a large number of entries with large numbers of replicates are needed. Non-random mating in a meadow fescue polycross with as many as 20 genotypes × 20 replicates was still detected by Myhre and Rognli (1990) by means of isozymes. The number of replicates is mostly above 10 to harvest enough seed for broadcast plot trials in several environments. Various PX designs have been suggested, mostly based on lattice or alpha designs, being well suited for small numbers of entries as in syn-0 nurseries (CE Wright 1962, Morgan 1988). For large numbers of entries, CE Wright (1965) developed field plans involving up to 50 clones. In practise, these plans have proven to be extremely valuable. Several variants of PX designs have been elaborated leading to reduced labour and costs. One is to plant replicated clonal rows instead of single plants. This reduces harvesting time and technical errors. Instead of clonal plants, individuals belonging to the same family can also be used for crops where vegetative propagation is difficult.

The *topcross test* (TX) was originally described to test large numbers of inbred line × variety crosses in maize (review by Hallauer and Miranda 1981). However, it also covers different forms of crosses between a number of candidates (clones, lines or families) and a common pollinator (tester). Surprisingly, in the forage breeding literature, an unrelated variety is still mentioned as the typical tester (Frandsen

et al. 1978, Nguyen and Sleper 1983, Sleper and Poehlman 2006). However, the tester could also be a related population like the breeding population from the previous cycle or even the base population. In both cases there will be enough seed to drill the tester rows. In crops like perennial ryegrass, where vegetative propagation is simple and cheap, a mixture of clonal parts could be used as the tester as well. In all cases where the tester is related to the candidates, genetic interpretation is identical to HS progenies derived from polycrossing. The use of an unrelated tester is used in *interpopulation* improvement.

The choice of tester depends largely on the stage of the breeding programme and varies from a population or synthetic, a single-cross hybrid to an inbred line, i.e. from genetically broad to narrow based. There have been many discussions about whether a tester should be strong or weak. These expressions refer to the genetic make up, and a weak tester is one carrying many recessive, negative alleles at loci responsible for the traits of interest. With a weak tester, the good performance of a progeny reflects the number of positive alleles of the particular candidate genotype.

In alfalfa and clover breeding, insect-proof cages can be established to isolate PX or TX nurseries and insect pollinators placed inside to provide for pollination. With wind-pollinating species spatial isolation of the PX or TX nursery is an important feature. Synchronization of the time of flowering is of fundamental importance and the difference between the earliest and latest flowering genotype should not exceed 5 days. In a topcross, the tester will provide an excess of pollen, which will predominate the pollen cloud.

The number of candidates in the PX or TX determines the number of families and consequently plots to be evaluated. HSF testing is mostly performed in drilled plots, replicated over locations and years and selection is on HSF means. The invention of forage plot harvesters enabled breeders to drastically increase the number of plots to be harvested. Under the assumption of a selection pressure of 10% and recombination targeted at 10–15 selection units, 100–150 genotypes have to be testcrossed and progeny tested. Depending on the species and the breeder's preferences, recombination can be done either with remnant seed or with the parental plants, if these have been maintained vegetatively. Recombination with remnant seed will reduce parental control and gain from selection to one-half.

Spaced Plants: Individual vs. Family Selection Assuming a spaced plant nursery with 10,000 individuals as in mass selection, then 100 HSFs with 100 plants/family could be tested. Ten families with 10 individuals per family may have been selected for recombination to generate 100 new HSFs. This results in a selection intensity of 10% ($i = 1.755$) for both among and within family selection, while in mass selection with 1% a 10-fold higher selection pressure can be achieved. Assuming equal single plant heritability, mass selection will be superior to AWHSF selection. England (1977) compared several types of family selection and his model calculations lead him to conclude that single plant plots are superior to replicated tests; in other words, that mass selection was the best method. These findings are supported by Gallais (1990) for both HS and FS *among and within* family selection. In an experimental study with HS progenies from two perennial ryegrass populations,

several combinations of selection pressure among and within families were compared and superiority of individual over family selection was observed (Charmet and Grand-Ravel 1991).

There are many published experiments demonstrating the success of spaced plant selection, whether mass or family selection. However, there are only a few experiments, where the response to selection was also evaluated under plot conditions. In perennial ryegrass, Ravel et al. (1995) concluded that individual plant selection failed to increase productivity under sward conditions, but produced some improvement in disease resistance and other individual plant-related traits. Similar results were found by Real et al. (2000) in diploid red clover.

To improve the generally poor relationship between individual plant and plot performance, Gallais (1977) and Wilkins (1991) suggested simultaneous evaluation under spaced plant as well as micro plot conditions.

Full-sib families (FSF) derive from paircrosses also known as bi-parental matings (BIPs). Since the parents (individual plants or clones) are non-inbred, FSFs are segregating. Under spaced plant conditions selection *among and within* full-sibs (AWFS) may be carried out. Testing FSFs under plot conditions is named full-sib family progeny test (FSPT). In self-incompatible grasses paircrosses are made by bagging together tillers from neighbouring plants without emasculation. With detached tillers as mentioned above, great flexibility in adjusting time of pollen shed can be achieved. However, to obtain enough seeds for plot trials parental clones are interplanted in an isolation in seed islands in a field of rye. If a paircross does not yield enough seed, an FSF could be multiplied under isolation to yield an FSF^2, which is partly inbred but still represents the gametic array of the two parental genotypes. The inbreeding coefficient F varies among FSF^2s, averaging to 0.25. Because FSF^2 are compared inter se, the inbreeding effect does not alter selection decisions. Recombination of the selected FSF^2 will result in a non-inbred population again.

The variance among FSF from non-inbred parents equals $\frac{1}{2}\sigma^2_A + \frac{1}{4}\sigma^2_D$, which corresponds with $2\ \sigma^2_{GCA} + \sigma^2_{SCA}$. The small fraction of nonadditive genetic variance can be largely ignored in practical breeding programmes. Theoretically, FSPT should be twice as effective as HSPT. However, to produce FSFs twice as many parents as with HSFs are needed and the production is more expensive. Assuming a selection pressure of only 10%, and a selected fraction of 25, 250 paircrosses have to be made. Recombination in FSF selection is based on selected plants or remnant seed of the selected families and could be done in two ways. As shown in Figure 7 (left), recombination among selected FSFs takes place in 1 year, while the paircrosses are made in a successive year. However, this procedure costs time and furthermore, the parentage cannot be controlled, and thus there is a risk of mating-related individuals. An alternative is shown on the right hand side of Figure 7. Recombination and production of new paircrosses can be performed as a single step if mating between relatives is prevented by controlled pollination. Recombination can be improved by increasing the number of crosses among unrelated families. Parentage and inbreeding can be controlled by applying partial diallel mating schemes or a series of factorial crosses.

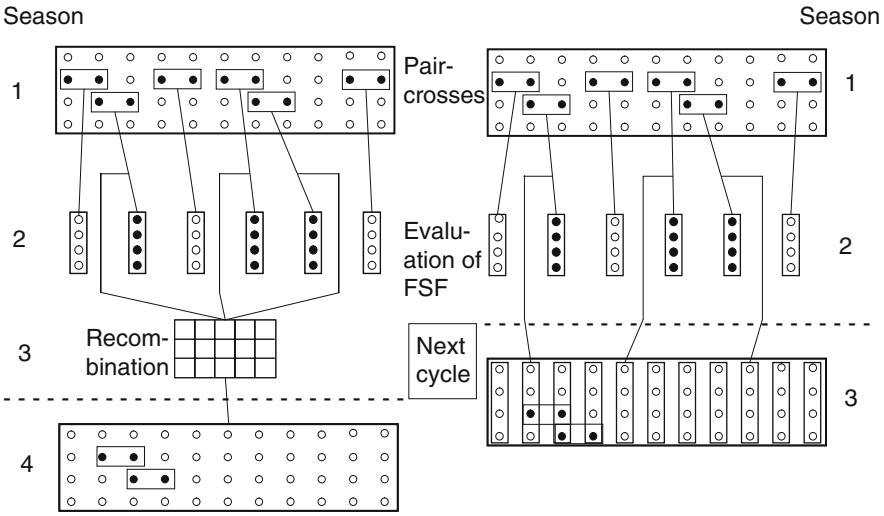

Fig. 7 Full-sib family selection with complete (*left*) and partial (*right*) recombination

S_1 families derive from selfing non-inbred parents and selection is among S_1 families. The variance among S_1 families from non-inbred parents is $\sigma^2_A + {}^1/_4\sigma^2_{D.}$, which equals $4\sigma^2_{GCA} + \sigma^2_{SCA}$. However, these estimates hold true only under the assumption of equal gene frequencies ($p=q$) and depend also on the degree of dominance. In general the genetic variance among S_1 families is clearly larger than the variance among testcrosses and thus, differentiation of the breeding material is superior. However, the S_1 performance is only an indirect selection criterion and what counts is a close genetic correlation between S_1 per se and testcross performance. In perennial ryegrass comparative plot trials (Posselt 1989b) the genetic correlation between S_1 and PX progenies was r_g=0.53. Simulation studies (Wright 1980) of various breeding procedures confirmed the superiority of S_1 testing in terms of response per cycle. Since bagged individual plants generally do not yield enough selfed seed for plot trials, they need to be cloned and transplanted into isolation facilities. Alternatively, a S_1 family can be multiplied in isolation to yield a $S_1{}^2$ family. The coefficient of inbreeding F will be somewhere between $F = 0.5$ (S_1) and $F = 0.75$ (S_2). For recombination, remnant seed of either S_1 or $S_1{}^2$ could be used. However, it has to be mentioned that S_1 and $S_1{}^2$ testing is a per se test, and the efficiency largely depends on the correlation between S_1 and testcross performance (GCA) of the respective parents (Posselt 1989b). If S_1 families are evaluated under spaced plant conditions varying degrees of inbreeding depression can be observed, which provide an indication of the sensitivity to inbreeding of the parents. This information is of relevance when selecting parents for building up synthetics. Under sward conditions competitive forces may lead to suppression or even elimination of weak plants (Charles 1966). In S_1 plot tests much lower inbreeding depression (about 15%) was observed as compared to about 40% under spaced plant

conditions (Posselt 1989b). Due to these selective forces the genetic variance among S_1 families was also much lower than theoretically expected.

2.2.3 Comparing Selection Methods

Parental Control In most forage crops and amenity grasses the parental material as well as the progenies can be maintained vegetatively. Additionally like in annual species, remnant seed is available for recombination. The amount of additive genetic variance is influenced by the parental control exercised in the recombination of selected individuals or families. "Parental control in recurrent selection is the relationship between the plant or seed used for identifying superior genotypes (selection unit) and the plant or seed used for recombination (recombination unit)" (Fehr 1987).

Parental control is $c = 0.5$ when the selection unit is the same as the recombination unit and only the female parent is selected, i.e. when selected female plants are pollinated by selected and unselected males in the population (i.e. control of one sex only). Parental control is 0.5 for recurrent phenotypic selection and maternal line selection when selection is done after pollination.

Parental control is 1 when the selection unit is the same as the recombination unit and both parents (i.e. control of both sexes) are selected. Parental control is 1 for recurrent phenotypic (mass) selection before pollination, for HSF selection when remnant HS seed is used for recombination, for FSF selection and for selfed families. This is also the case when selected individuals from AWF selection are removed from the field for recombination.

Parental control is 2 when the selection and recombination units are not the same. Parental control is 2 for HSF selection when selfed seeds or clones of selected genotypes (parental unit) are used for recombination after a progeny test carried out with plants raised from HS seed. In this case, the selection unit is HS seed, but the recombination unit is selfed seed or clones of selected genotypes (Fehr 1987).

Recombination and Inbreeding The importance of the effective population size in respect to inbreeding was shown in Table 2. In family selection N_e is different from phenotypic selection due to the genetic relationship among related individuals. N_e depends on the inbreeding coefficient of the recombination unit (Gordillo and Geiger 2008). Each further step of selection further reduces N_e. Generally, to get the same effective population size and thus a comparable level of inbreeding, the number of parents must show a ratio of 1:2:4:8 when using HSF, FSF, clones (or single crosses of inbred lines or S_1 lines) and homozygous inbred lines, respectively (Becker 1988).

The most common selection methods are summarized in Table 3. HSF-progeny testing in replicated plot trials and recombination of remnant HS seed by polycrossing is probably the most popular method in practical breeding. Of course, different procedures can be used simultaneously or successively in a population improvement programme.

Table 3 Intrapopulation improvement methods[1]

Method	SU	TU	RU	c	y	σ^2_{bG}		σ^2_{wG}	
						σ^2_A	σ^2_D	σ^2_A	σ^2_D
Mass af	Pl	Pl	POP	0.5	2	1	1	0	0
Mass bf	Pl	Pl	Pl	1	2	1	1	0	0
HS_{AWF}	Pl	Pl	HS_{Plant}	1	5	1/4	0	3/4	1
HS_{PT}	Fam	Plot	HS_{Seed}	1	5	1/4	0	3/4 §	1 §
HS_{PT}	Fam	Plot	HS_{Parent}	2	5	1/4	0	3/4 §	1 §
FS_{AWF}	Pl	Pl	FS_{Plant}	1	5	1/2	1/4	1/2	3/4
FS_{PT}	Fam	Plot	FS_{Seed}	1	5	1/2	1/4	1/2 §	3/4 §
S_1	Pl	Pl	S_{1Plant}	1	5	1*	1/4*	1/2*	1/2*

[1]SU – selection unit, TU – test/evaluation unit, RU – recombination unit, c – parental control, y – number of years per cycle, $\sigma^2_{bG\ and\ wG}$ – genetic variance between and within families, af and bf – after and before flowering, Pl – plant, POP – population, Fam – family; §not usable, *approximate values if allele frequencies deviate from 0.5.

While comparing HSF and FSF selection, Hallauer and Miranda (1981) showed that comparisons among selection systems could be done either on N_e for equality of i (selection intensity) or based on equal N_e with adjusted i. With equal i (same number of test plots) N_e is twice as large in HSF as compared to FSF. The latter approach was chosen by Casler and Brummer (2008) and is considered as being adequate for short-term selection (Hallauer and Miranda 1981).

For long-term selection the choice of an equal effective population size is recommended. Under the restriction of equal N_e, about twice as many FSFs are needed for recombination in comparison to HSFs, and the respective family size in HSFs should be twice of that of FSFs (Hallauer and Miranda 1981).

For long-term selection programmes, N_e should range between 25 and 50 to comply with an upper limit of the inbreeding rate of $F = 0.01$ (Crow and Kimura 1970). According to the ratios given above, this corresponds to about 7–13 HSFs, 13–25 FSFs or 25–50 clones (Geiger and Miedaner 2009).

A criterion for the efficiency of alternative breeding strategies is the amount of improvement or "genetic gain" realized per year. Parameter estimates needed for calculating expected response from most intrapopulation selection systems are additive genetic variance (σ^2_A) in the population to be improved and the total phenotypic variance (σ^2_{ph}) among individuals or among families being selected.

In family selection systems the breeder can raise the heritability by increasing the number of test environments and/or replicates per environment. Using the formula in Table 5, and taking into consideration the costs of testing, then an optimum resource allocation can be found. With a given budget, a certain number of plots can be tested. From the formula in Table 4 and the model calculations in Table 5 it is obvious that increasing the number of test locations is more profitable than an increase in number of replicates.

Table 4 Expected gain per cycle from intrapopulation selection systems with non-inbred parents (adapted from Fehr 1987)

Method of selection	Expected gain per cycle (G_c)[1]
Mass	$\frac{kc\sigma_A^2}{\sqrt{\sigma_u^2 + \sigma^2 + \sigma_{AE}^2 + \sigma_{DE}^2 + \sigma_A^2 + \sigma_D^2}}$
Half-sib family	$\frac{kc\frac{1}{4}\sigma_A^2}{\sqrt{\frac{\sigma_e^2}{rt} + \frac{\frac{1}{4}\sigma_{AE}^2}{t} + \frac{1}{4}\sigma_A^2}}$
Full-sib family	$\frac{kc\frac{1}{2}\sigma_A^2}{\sqrt{\frac{\sigma_e^2}{rt} + \frac{(\frac{1}{2}\sigma_{AE}^2 + \frac{1}{4}\sigma_{DE}^2)}{t} + \frac{1}{2}\sigma_A^2 + \frac{1}{4}\sigma_D^2}}$
S_1 family	$\frac{kc\sigma_{A^*}^2}{\sqrt{\frac{\sigma_e^2}{rt} + \frac{(\sigma_{AE^*}^2 + \frac{1}{4}\sigma_{DE^*}^2)}{t} + \sigma_{A^*}^2 + \frac{1}{4}\sigma_{D^*}^2}}$

[1] k selection intensity in standard units, $\sigma^2{}_u$ is the within-plot environmental variance, $\sigma^2{}_{AE}$ and $\sigma^2{}_{DE}$ are the additive by environmental and dominance by environmental interaction variances, $\sigma^2{}_A$ and $\sigma^2{}_D$ are the additive and dominance variance, r is the number of replications per environment, t is the number of environments. $\sigma^2_{A^* \text{ or } D^*}$ as in Table 3.

Table 5 Expected gain from HS family selection for three levels of selection intensity (%) and a given number of replicates and test locations (Posselt 2003a)

		Selection intensity		
Location	Replicates	5%	10%	20%
1	2	5.4	4.6	3.6
2	2	7.4	6.4	5.0
2	4	8.2	7.6	5.5
3	2	8.8	7.8	5.9
3	4	9.7	8.3	6.5

G×E Interaction Because of the great ecological variation among target environments genotype × environment interactions (G × E) are of special interest to the breeder. Especially in stress environments large G × Y interactions due to carry-over effects can be observed. In the two examples listed in Table 6, G × Y interaction was more important than G × L.

The value of any kind of prediction (genetic gain, synthetic or hybrid performance) strongly depends on the precision and accuracy of the estimated parameters. The large differences in the genotypic variance components between the two series in Table 6 demonstrate how far variance component estimates may vary between experiments even if conducted in the same environments.

Table 6 Variance components for annual dry matter yield in two HS-progeny tests at two locations over 3 years (Posselt 2000)

	Variance component estimates	
Source of variation	Narrow population	Broad population
Genotype (G)	0.117	0.499
G × location (G × L)	0.102	0.049
G × year (G × Y)	0.172	0.291
G × L × Y*	0.168	0.233

*confounded with error.

2.3 Creation of Open-Pollinated Varieties and Synthetics

2.3.1 Open-Pollinated Varieties

After several cycles of recurrent selection the improved breeding population will be assessed not only for spaced plant characters, but also for population performance under sward conditions in comparison with released varieties. If the performance level is competitive, the improved population can be used to create an OPV. At this point the breeder has to consider DUS (distinctness, uniformity, stability) regulations. In most cases phenotypic variation is too large to comply with uniformity (homogeneity) requirements. In the case of time of flowering phenotypic variation is reduced by discarding, for instance, too early and too late flowering plants. If no genetic correlation exists between DUS characters and target traits this will not affect trait performance.

In general at least 100 individual genotypes or the best families from the last RS cycle will be intermated to produce the new OPV. The progeny will be planted with large numbers as spaced plants to check for uniformity again. Off-types will be eliminated to keep the population true-to-type. Seed will be bulked after harvest. This seed lot serves for (i) DUS and VCU (value for cultivation and use) testing, (ii) as breeders seed in seed multiplication and (iii) as nucleus in variety maintenance.

In the strict sense, an OPV is maintained by periodically repeating the procedure above, thereby allowing for a continuous development without violating the stability requirement of DUS. Nowadays, breeders prefer maintaining OPVs by storing seed reserves under adequate conditions. This serves to limit the number of generations in seed multiplication and minimizes the risk of violating DUS requirements.

2.3.2 Synthetic Varieties

A basic scheme for synthetic breeding is given in Figure 8. Potential parents are selected from an improved breeding population. In perennials, clonal line evaluation can be practised as a second step of parent selection. According to practical experience, at least one-third of the clones have to be discarded because of their inferior performance. The production of testcrosses and their testing is indispensable to estimate GCA values. The progeny test is of utmost importance to obtain

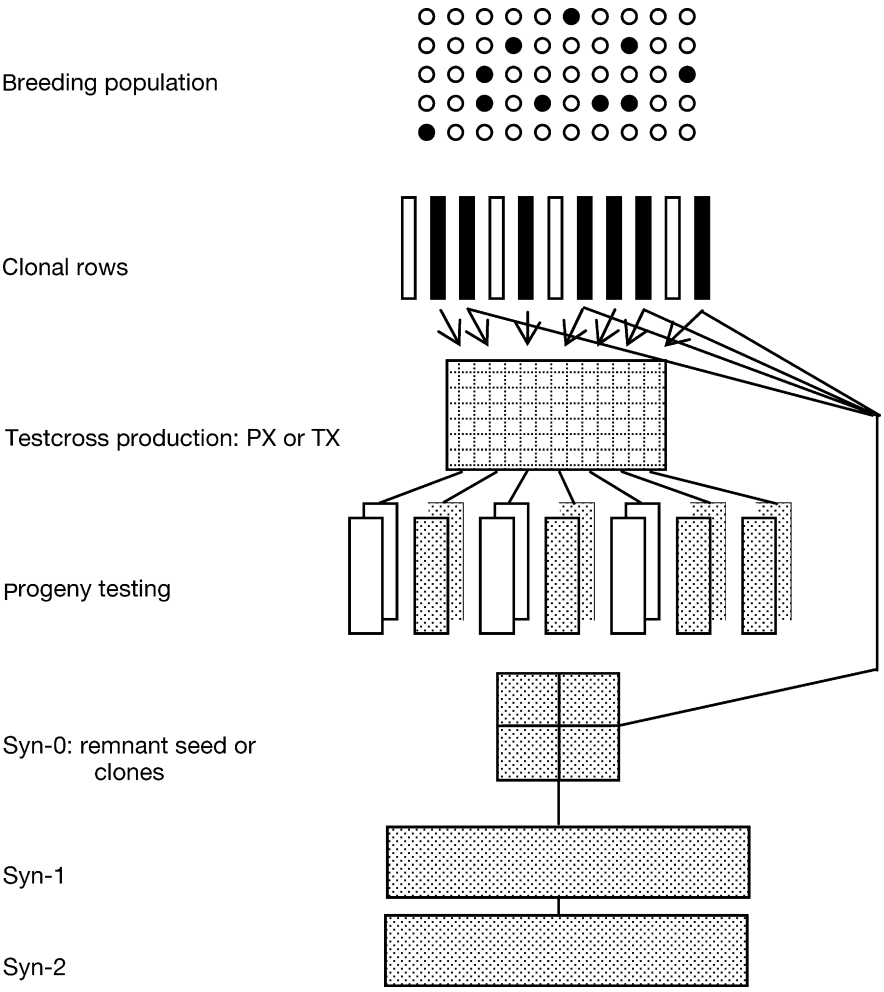

Fig. 8 Breeding scheme for developing synthetic varieties

reliable data for predicting superior experimental synthetics. A minimum testing at two locations over 2 or 3 years is recommended. In this context, the breeder should choose the same testing system in terms of time and number of cuts as applied in the VCU trials. The final choice of the parents should be based on synthetic prediction as described in the following section. Additionally, DUS requirements have to be taken into account. As indicated in Figure 8, synthesis can be carried out from parental clones or remnant seed from testcrossing. Because of improved seed storage facilities many breeders prefer the remnant seed option. In this system additional selection for morphological traits within the progenies is practised before intermating. This may partially counterbalance the loss of parental control by using remnant seed instead of the parental clones.

Random intercrossing generally is a very difficult step in synthetic production due to unequal pollen shed and/or seed set of the parents. A partial solution to these problems is to harvest Syn-1 seed separately from each parent component and then mix equal quantities of seed before further multiplication. When using clones, diallel matings with seed adjustment among the paircrosses and a well-designed mating scheme like in polycrossing will improve random pollination in Syn-2 production. Syn-2 is mostly produced by broadcasting Syn-1 seed. In diploids the Syn-2 is in equilibrium and will be the earliest generation for VCU testing. For commercialization further seed multiplication is undertaken and the Syn-4 will mostly serve as certified seed.

Variety maintenance can be done in several ways:

- maintenance of the parental clones (in situ or *in vitro*) and reconstitution of the synthetic as needed;
- production of a large amount of Syn-1 seed for cold storage. Further multiplication as needed;
- Syn-2 reserve in cold storage.

Before synthetics became popular OPVs were predominant. Nowadays, under competitive market conditions, at least in the economically important species, OPVs can no longer compete with synthetics. However, in new or minor species the breeding of OPVs is still of interest, especially, because of lower input in terms of costs compared to synthetic breeding. In general, within OPVs a large amount of useful variation is still available. Without violating DUS, an OPV can be further improved for agronomically important traits like seed yield. This was done in the past applying the so-called "amelioration breeding". Unfortunately, these seed yield improved OPVs often showed reduced persistence and biomass yield.

2.3.3 Prediction of Synthetic Varieties

The theoretical framework for predicting the performance of synthetics traces back to Sewall Wright in 1922, who presented the formula given in most plant breeding textbooks:

$$\widehat{F}_2 = \overline{F}_1 - \frac{\left(\overline{F}_1 - \overline{P}\right)}{n} \tag{3.2}$$

where F_2 represents the estimated performance of the F_2 generation, F_1 is the average performance of all possible single crosses among the parental lines involved, P is the average performance of the parents and n the number of parents involved (Allard 1960, Fehr 1987). Unfortunately, Sewall Wright used the term ". . .derived from inbred families. . ." and in many textbooks P is still defined as the average performance of homozygous inbred lines. The formula is valid when using inbred lines like those in maize, but for non-inbred materials as is the practise in forage crops, P has to be replaced by S_1, the selfed progeny of the parents. It is therefore more

convenient to use the formula: $Y=C-[(C-S)/n]$, with Y being the expected yield of a synthetic in equilibrium. C is the mean of all possible crosses between n parents and S is the mean of all intra-parent progenies (i.e. S_1 per se). The term in brackets refers to the inbreeding depression which occurs during seed multiplication from Syn-1 to Syn-2 due to intermating of related plants. It also has to be mentioned that the formula assumes allogamy, absence of selection, absence of epistasis and diploidy.

For polyploids, Gallais (1976) presented a generalized Sewall Wright formula including a term which accounts for the level of ploidy:

$$\widehat{Y} = C - \frac{2k-1}{k} \times \frac{C-S}{n} \tag{3.3}$$

with k=1, 2 or 3 for diploids, tetraploids or hexaploids, respectively.

A more advanced theory including polyploidy, epistasis and partial to complete self-fertilization has been elaborated mainly by Busbice (1969, 1970), Gallais (1974, 1975, 1976) and Wright (1974, 1981). The theory of synthetic breeding is also presented in the textbooks of Wricke and Weber (1986) and Gallais (1990). The interested reader is referred to the more applied review elaborated by Becker (1988). Synthetic prediction based on experimental data of perennial ryegrass was presented by Posselt (1984a, 2000).

According to current terminology the parent generation of a synthetic is designated as Syn-0. The composition of the next generation (Syn-1) is likewise a diallel, with selfs on the diagonal (Table 7). Gilmore (1969) was the first to show that Wright's formula is valid for synthetic prediction, whether the parents are inbred or not, and that the random combination of four heterozygous plants (S_0) or their S_1 progeny, or eight conceptual inbred lines will give identical results. The formula is also valid when instead of S_0 clones, HSF or FSF or even narrow populations are used as parents. From Table 8 it is obvious that data from diallel crosses among heterozygous clones will perfectly supply all the information needed for synthetic prediction.

If all n parents are synthesized, then the grand total of the diallel will be a good predictor of the Syn-2(n). In the textbooks of Wricke and Weber (1986) and Gallais (1990), the size (number of parents) of a synthetic in equilibrium is designated as k-Syn-e, which in self-incompatible materials is equal to the Syn-2 generation. In

Table 7 Scheme of the genetic composition of Syn-1

Parent	P1	P2	...	Pn
P1	Y_{11}	Y_{12}		Y_{1n}
P2	Y_{21}	Y_{22}		Y_{2n}
⋮	⋮	⋮	⋮	⋮
Pn	Y_{n1}	Y_{n2}		Y_{nn}

Table 8 Synthetic prediction ($\hat{Y}_{GCA}$) of the best synthetics of size n according to formula (3.4), based on the topcross performance of 35 clones and their combining ability (GCA) in Mg ha^{-1}. S is assumed as 80% of C (Posselt 2000)

	Topcross performance			Prediction	
Clone	Mean	GCA	No. of parents	$\mu(n)$	$\hat{Y}_{GCA}$
1	12.10	1.26	–	–	–
2	11.89	1.05	2	9.75	10.89
3	11.85	1.01	3	10.11	11.53
4	11.82	0.98	4	10.29	11.80
5	11.75	0.91	5	10.40	12.02
6	11.64	0.80	6	10.47	12.15
7	11.64	0.80	7	10.52	12.21
8	11.58	0.74	8	10.56	12.23
9	11.52	0.68	9	10.59	12.23
10	11.43	0.59	10	10.61	12.22
11	11.41	0.57	11	10.63	12.20
12	11.38	0.54	12	10.65	12.18
13	11.38	0.54	13	10.66	12.17
14	11.31	0.47	14	10.68	12.14
15	11.23	0.39	15	10.69	12.11
16	11.13	0.29	16	10.70	12.07
17	11.03	0.19	17	10.70	12.03
18	10.72	−0.11	18	10.71	11.95
19	10.72	−0.11	19	10.72	11.89
20	10.65	−0.18	20	10.72	11.82
21	10.59	−0.24	21	10.73	11.75
22	10.54	−0.29	22	10.73	11.69
23	10.42	−0.41	23	10.74	11.62
24	10.28	−0.55	24	10.74	11.55
25	10.26	−0.57	25	10.74	11.47
26	10.17	−0.66	26	10.75	11.40
27	10.13	−0.70	27	10.75	11.33
28	10.07	−0.76	28	10.75	11.26
29	10.06	−0.77	29	10.76	11.20
30	9.89	−0.94	30	10.76	11.12
31	9.88	−0.95	31	10.76	11.06
32	9.76	−1.07	32	10.76	10.98
33	9.75	−1.08	33	10.76	10.92
34	9.69	−1.14	34	10.77	10.85
35	9.40	−1.43	35	10.77	10.77

this chapter we will use n instead of k, and the size of the synthetic is given in brackets. Thus a six-parent synthetic in the third generation is given as Syn-3(6).

According to the diallel formula [$n \times (n-1)/2$] it is obvious that from 10 parents 45 two-parent synthetics [Syn-2(2)] could be composed. However, there could be also various synthetics with 3–10 parents. The number of all possible synthetics can be calculated according to $2^n - n - 1$ (Wricke and Weber 1986). With 10 parents 1013 possible synthetics could be produced. From these numbers it is quite clear that it is

not practical to synthesize all of them. Therefore the prediction of the performance of all possible synthetics is of paramount importance to the breeder and synthetic testing can be limited to the most promising ones.

Although the most complete evaluation of parents is to intercross them in a diallel fashion, this laborious design where only low numbers of parents could be tested, is not applicable in practical breeding. Fortunately, the theory of synthetic prediction has provided breeders with alternative options. Thus, the parents of a synthetic variety could be selected according to the information available: (i) based on GCA only, (ii) using additional information of S_1 performance and (iii) including SCA, if available. The prediction based on only GCA estimates is the most relevant approach for practical breeding and will be discussed in more detail.

Synthetic performance for a selected number of diploid, non-inbred clones can be predicted by a mean value μ plus the weighted sum of GCA:

$$\widehat{Y}_{\mathrm{GCA}} = \mu(n) + \frac{2(n-1)}{n^2} \sum_i \mathrm{GCA_i}; \tag{3.4}$$

with

$$\mu(n) = C - \frac{C - S}{n}$$

which is the mean of all synthetics of size n. If all parents available are used, then the sum of all GCA-values becomes 0 and thus equation (3.4) reduces to $\mu(n)$. If S is not available from experimental data, as is mostly the case, an estimated value (S=x% of C) can be used (Posselt 1984a, Wricke and Weber 1986). In perennial ryegrass plot trials the average S_1 performance was about 85% of the testcross mean (Posselt 1984a). However, there are two points to be made: (i) there is large genetic variation in inbreeding depression and (ii) there is a positive correlation between S_1 performance per se and GCA of the parental clones. In any case, parents less sensitive to inbreeding should be preferred.

Experimental data from a 35-clone TX-test (see Table 8) were used to predict the mean ($\mu(n)$) and the performance ($\hat{Y}_{\mathrm{GCA}}$) of all synthetics of size n based on GCA. Inbreeding depression was assumed to be S=80% of C, which is the mean performance of the respective clones. From formula (3.4) one can deduce that because of the inbreeding effects the number of parents should be large, while on the other hand GCA effects demand for low n. According to Figure 9 the near-optimum domain is between 7 and 11 clones. With only 2–4 parents, the positive GCA effects are counterbalanced by the opposite effect of inbreeding. With larger numbers of clones GCA values of added clones become more important than the beneficial effect on decreasing inbreeding. If a breeder uses all parents available then this situation is identical to the creation of an OPV and it is obvious from Figure 9 that the OPV is inferior to the best possible synthetic.

A large number of experimental investigations on the optimum number of parents can be found in the literature and have been summarized by Becker (1988). In most experiments the optimum number of components was 5, and the use of more

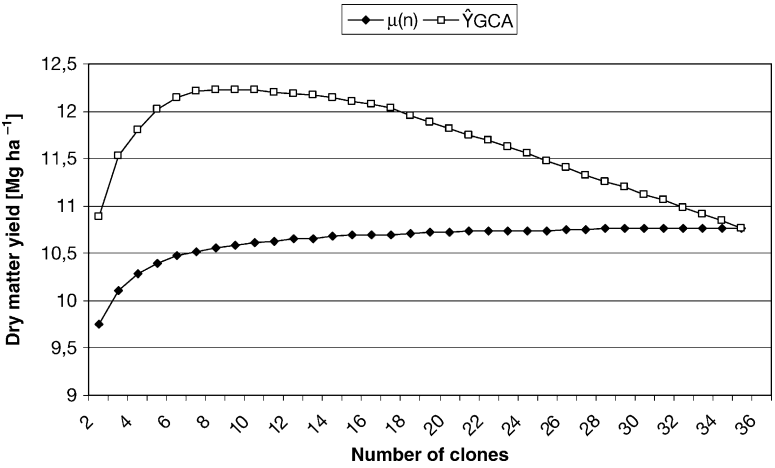

Fig. 9 Prediction of synthetic performance ($\hat{Y}_{GCA}$) based on the data of Table 8. *S* is assumed to be 80% of *C*

than 10 clones was never recommended. However, according to Becker (1988), in several of these experiments only synthetics with 2–6 clones were tested, and thus it is not surprising that the "optimum" number recommended was the largest number investigated experimentally. It has to be emphasized that the optimum number of parents mainly depends on the material under study and also on the number of parents tested.

The influence of the degree of inbreeding depression on synthetic prediction is shown in Figure 10. *S* is assumed to be 90, 80 or 70% of *C*, and the predicted synthetics are designated accordingly as $\hat{Y}(90)$, $\hat{Y}(80)$ and $\hat{Y}(70)$, i.e. from small to larger

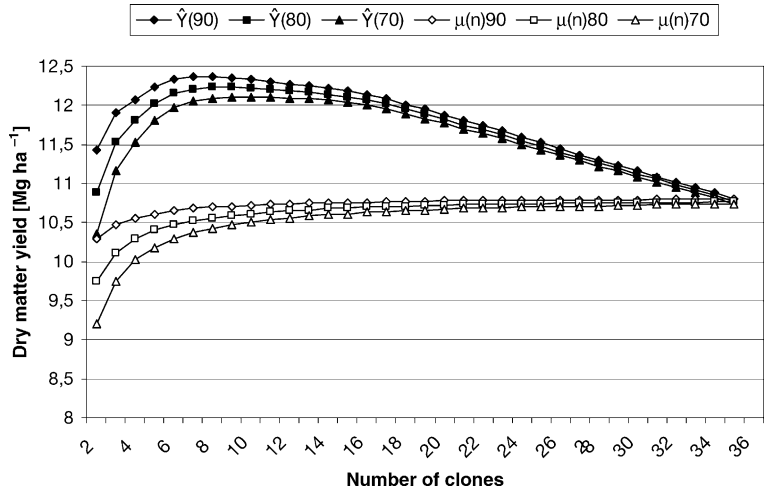

Fig. 10 Synthetic prediction for several degrees of inbreeding (see text)

inbreeding depression. The effect of different degrees of inbreeding depression is very striking and most pronounced for low number of clones. As expected, inbreeding decreases the performance of the predicted synthetics: $\hat{Y}(90) > \hat{Y}(80) > \hat{Y}(70)$. However, the most marked effect is on the optimum number of parents. First, the optimum is more pronounced for low inbreeding [$\hat{Y}(90)$] and rather flat for $\hat{Y}(70)$. Second, there is a clear shift in the optimum number of parents from 7 or 8 to 9 or 10 from $\hat{Y}(90)$ to $\hat{Y}(70)$. Thus, with inbreeding sensitive materials higher numbers of synthetic parents are needed.

According to Figures 9 and 10, the optimum is a range of very similarly performing synthetics composed from 7 to 11 clones. The breeder could (i) synthesize several of these synthetics based on prediction and compare the Syn-2(n) in performance trials, (ii) use additional information of morphological data relevant for DUS or (iii) use additional trait data. It should also be stressed that the accuracy of the prediction depends on the experimental error of the test in which GCA values were determined. This is particularly true for progeny tests carried out in a single environment, not taking into account G × E interaction.

2.3.4 Information on Selfed Progeny Is Available

In many species selfing is possible and instead of an estimate of S the performance of the selfed progeny (S_1, if the parents are non-inbred clones) can be estimated directly. The S_1 performance is considered in the prediction of the general varietal ability (GVA). The GVA (*syn*. GSA, general synthesizing ability, as used by Wricke and Weber 1986 and Gallais 1990) of a clone is the mean of all synthetics of size n to which this parent contributes. Thus, GVA values vary with the size of the synthetic, while GCA values are independent from n. As can be seen from the formula (3.5), GVA is a combination of GCA and S_1 performance and the importance of the latter increases as n decreases. The concept of GVA was developed by Wright (1974) and extended to polyploids by Gallais (1975).

$$\begin{aligned} &\mathrm{GVA}(n)_\mathrm{i} = \tfrac{1}{n}\left(2\ \mathrm{GCA}_\mathrm{i} - \frac{2\ \mathrm{GCA}_\mathrm{i} - L_\mathrm{i}}{n}\right), \quad \text{with } L_\mathrm{i} = S_\mathrm{i} - \overline{S}, \text{ and} \\ &\hat{Y}_\mathrm{GVA} = \mu(n) + \sum_i \mathrm{GVA}(n)_\mathrm{i} \end{aligned} \tag{3.5}$$

L_i is the deviation of the S_1 performance of clone i from the mean of all selfs

From Figure11 based on the data from Table 9, it is obvious that only for low number synthetics $\hat{Y}_\mathrm{GVA}$ is slightly superior to $\hat{Y}_\mathrm{GCA}$. However, for both prediction methods the optimum number of clones for this particular material is four. The optimum of the prediction curves is rather flat as compared to the curves in Figure 10. This is due to a much lower genetic variance among the 22 clones in comparison to the 35 clones, where the estimated genetic variance was four times larger (data not shown). As expected the optimal number of parents is smaller than in the previous case since fewer (22 vs. 35) potential parents were evaluated.

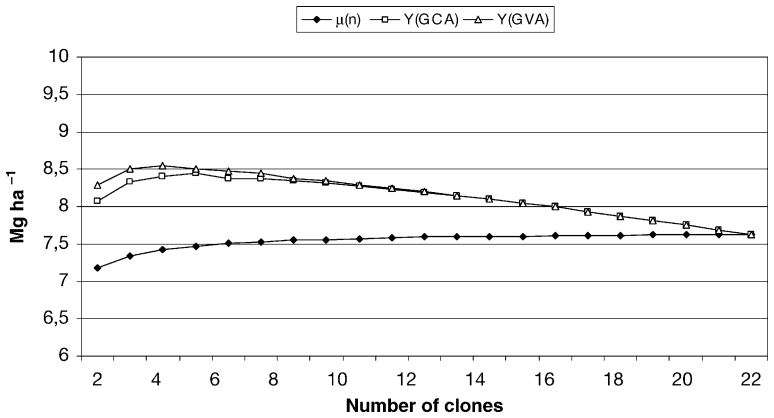

Fig. 11 Prediction of synthetic performance based on the data of Table 9 (for details see text)

Table 9 Performance (in Mg ha^{-1}) of 22 clones in crosses (Ci and GCAi), their selfed progeny (S_i and L_i) and prediction of $\mu(n)$, $\hat{Y}_{\mathrm{GCA}}$ and $\hat{Y}_{\mathrm{GVA}}$ according to formula (3.6) (Posselt unpublished)

	Crosses		Selfs		Prediction		
Clone	C_i	GCA$_i$	S_i	L_i	$\mu(n)$	$\hat{Y}_{\mathrm{GCA}}$	$\hat{Y}_{\mathrm{GVA}}$
1	8.70	1.30	6.93	0.23			
2	8.41	0.74	6.79	0.09	7.18	8.07	8.29
3	8.12	0.45	7.91	1.21	7.34	8.33	8.50
4	8.08	0.41	7.28	0.58	7.43	8.41	8.55
5	8.04	0.37	6.38	−0.31	7.47	8.44	8.51
6	7.94	0.27	6.70	0.01	7.51	8.38	8.47
7	7.88	0.21	6.02	−0.67	7.53	8.38	8.44
8	7.87	0.20	7.49	0.79	7.55	8.35	8.38
9	7.84	0.17	6.55	−0.14	7.56	8.32	8.35
10	7.72	0.05	6.79	0.09	7.57	8.28	8.29
11	7.68	0.01	5.88	−0.81	7.58	8.23	8.25
12	7.66	0.00	7.23	0.53	7.59	8.19	8.20
13	7.65	−0.01	6.23	−0.46	7.59	8.15	8.15
14	7.52	−0.14	6.72	0.02	7.60	8.10	8.10
15	7.48	−0.18	7.02	0.32	7.60	8.05	8.05
16	7.43	−0.23	6.65	−0.04	7.61	8.00	8.00
17	7.23	−0.43	6.36	−0.33	7.61	7.93	7.93
18	7.21	−0.45	6.16	−0.53	7.61	7.87	7.87
19	7.17	−0.49	6.38	−0.31	7.62	7.81	7.81
20	7.07	−0.59	6.74	0.04	7.62	7.75	7.75
21	7.01	−0.65	6.79	0.09	7.62	7.68	7.68
22	7.00	−0.66	6.27	−0.42	7.62	7.62	7.62

2.3.5 Information on SCA Is Available

Analogous to the concept of GCA and SCA, the general varietal ability GVA can be supplemented by an effect of the specific varietal ability (SVA). The SVA of the combinations of parents i and j for a synthetic of size n is

$$\begin{aligned} \mathrm{SVA}(n)_{ij} &= \frac{1}{n^2}\, 2\ \mathrm{SCA}_{ij} \quad \text{and} \\ Y_{\mathrm{SVA}} &= \mu(n) + \Sigma\ \mathrm{GVA}(n)_i + \Sigma\ \mathrm{SVA}(n)_{ij} \end{aligned} \tag{3.6}$$

Based on the effects of $\mathrm{GCA_i}$, L_i and SCA_{ij}, the prediction formula is

$$\hat{Y}_{\mathrm{SVA}} = \mu(n) + \frac{2(n-1)}{n^2} \Sigma\ \mathrm{GCA}_i + \frac{1}{n^2}\left(\Sigma L_{\mathrm{i}} + 2\ \Sigma\ \mathrm{SCA}_{ij}\ \right) \tag{3.7}$$

As can be seen from formula (3.8), the coefficient of the term ($\sum L_i$+2$\sum \mathrm{SCA}_{ij}$) is $1/n^2$, which means that GVA is nearly completely determined by GCA unless n is very small. Becker (1988) concluded that the parents for a synthetic variety can be reliable selected by testing their GCA only, if n is larger than about 5. To obtain reliable estimates for L_i and SCA additional efforts in mating and testing is required and it is questionable if the additional information will counterbalance these efforts.

So far the optimum number of components was discussed with special emphasis on forage yield performance, but there are other aspects to be considered. Phenotypic similarity of clones is necessary to satisfy legislative DUS requirements, and this will often restrict the number of clones available. In synthetic varieties constructed from very few parents yield stability or adaptation might be inferior to broad-based synthetics (Becker 1988). In very small synthetics unpredictable changes from generation to generation were observed, as well in yield (Vincourt 1981) as in morphological appearance (Grundler 1984). These changes could be due to mistakes in building up the synthetics (unequal amounts of seeds among the parents) and/or epistasis (Wright 1981). Gallais (1975) presented a formula based on two-parent synthetics in equilibirium (syn-e(2)), which gave a useful prediction valid in the presence of both dominance and additive × additive epistasis. This formula could be applied in FSF selection schemes where FSF^2 are tested. The effect of epistasis in advanced generations can be positive or negative. A yield decrease due to epistasis might occur if parents from genetically very distinct populations are combined and if the superiority of these source populations is partly due to favourable epistatic effects (Becker 1988).

2.3.6 Synthesis and Further Multiplication

Two different possibilities of Syn-1 production have to be considered:

(i) *Random intercrossing* of the *n* parents grown as a bulk under pollen isolation. Under the assumption of panmictic conditions, the Syn-1 will already be in Hardy–Weinberg equilibrium.
(ii) *Controlled crossing*, which means that self-fertilization and crosses between plants of the same parent are excluded. This is the case if the parents are intermated manually. In self-incompatible crops this is possible without emasculation. This is the typical case in most forage crops or amenity grasses. Equilibrium is reached in Syn-2 after one generation of multiplication by open

pollination. In most forage crops a slight yield decrease from Syn-1 to Syn-2 is observed. This decline is proportional to the reduction in heterozygosity due to the fact that with complete self-incompatibility, no inbreeding occurs in Syn-1 production, while a certain degree of inbreeding results from intermating of related plants in the further generations. The theoretical extent of the decline is described by the term $(C-S)/n$ as explained under "Prediction of synthetic varieties". Neglecting epistasis, the expected depression is equal to one-half of the heterotic increase from Syn-0 to Syn-1. Working with Italian ryegrass and meadow fescue, Vincourt (1981) showed that this decline was much more pronounced under spaced plant conditions than in dense swards. In a broadly based study experimental synthetics of perennial ryegrass from breeders in Belgium, Germany and the Netherlands were tested at three locations for 2 years. The Syn-2/Syn-1 comparison revealed a 2% superiority of the Syn-1 (Reheul et al. 2003).

Generally the performance of a diploid synthetic should be constant after one generation of random mating. This was confirmed by most experimental data (for review see Becker 1988). The most critical assumptions are absence of epistasis and natural selection during seed multiplication.

2.3.7 Self-Fertility

In many fodder crops and amenity grasses the production of inbred lines is hampered by self-incompatibility. However, as was shown by Posselt (1982) genes for self-fertility exist in many populations and self-fertile inbred lines were developed at Hohenheim in perennial ryegrass (Utz and Oettler 1978). In spring rye it was observed that synthetics from self-fertile materials yielded 10–15% less than comparable synthetics of self-incompatible material (Singh et al. 1984). The average amount of self-pollination in open-pollinated self-fertile rye populations was estimated to range between 35 and 40%. For the progenies of crosses among the above mentioned self-fertile inbred lines of perennial ryegrass, self-pollination rates of 0–63% were observed (Posselt, unpublished data). In conclusion, *the use of self-fertile material should strictly be avoided in breeding synthetics because complete outcrossing cannot be guarantied.*

Doubled Haploids (DH) In several grass species it was possible to develop DH-lines via anther culture at Hohenheim. However, the rate of success was highly genotype dependent. Moreover in a large number of perennial ryegrass DHs no seed set after selfing could be obtained. These DH plants were therefore strictly self-incompatible. However, DHs can be easily maintained vegetatively. From a set of five unrelated donor plants two DH-lines were obtained from each of them. Two types of synthetics were synthesized: (i) a five-clone synthetic and (ii) a 10 DH-line synthetic. In comparative field experiments of the Syn-2 over 2 years at two locations, a slight but not significant superiority of the DH synthetic was observed at a yield level of about 12 Mg ha^{-1} of annual dry matter yield (ADMY). A possible

explanation for this (Röber et al. 2005) is that DH-lines carry hardly any genetic load due to a strong selection pressure against deleterious recessive genes in the haplophase. Further research on the use of DHs in forage plant breeding is needed.

3 Hybrid Breeding

Hybrids derive from controlled matings of two parent components *i* and *j* and allow the breeder (i) to fully exploit the panmictic-midparent heterosis (PMPH; Lamkey and Edwards 1999) of crosses between genetically distant populations (heterotic groups) and (ii) to capitalize not only on GCA but also on SCA effects (Geiger and Miedaner 2009). The genotypic value of a hybrid can be described as $Y_{i \times j} = m + GCA_i + GCA_j + SCA_{ij}$, where $Y_{i \times j}$ is the hybrid performance, m is the mean of all crosses, GCA_i and GCA_j are the GCA effects of the parents i and j and SCA_{ij} is the specific combining ability of the two respective parents.

Hybrid breeding demands (i) identical reproduction and multiplication of the parental components on a large scale, (ii) controlled crossing of parents on a large scale and (iii) fertility of hybrids if seeds are to be harvested. Biological prerequisites are (i) a sufficiently high degree of heterosis for economically important traits and (ii) a suitable hybridization mechanism. Because of the flowering biology of forage crops, at present only CMS (cytoplasmic genic male sterility) and SI (self-incompatibility) are suitable hybridization systems (Table 10).

Table 10 Types of hybrids

Designation	Mating scheme[1]	Abbreviation
Population∼	POP' × POP''	PC
Topcross∼	(A × B) × POP''	TC
Double cross∼	(A × B) × (R × S)	DC
Three way∼	(A × B) × R	TW
Single cross∼	A × R	SC

[1]POP', POP'' = OPVs or synthetics; A, B and R, S are inbred lines from POP'and POP'', respectively

Uniformity increases from population (PC) to single-cross (SC) hybrids. In SCs based on inbred lines both heterozygosity and heterosis can be maximized. In self-incompatible species like fodder crops and amenity grasses, a single cross from two non-inbred parents [(AB) × (RS)] corresponds with a DC and is sometimes referred to as "cryptic double". Due to its genetic constitution it is a segregating F_1.

3.1 The Concept of Heterosis

The terms hybrids and heterosis are sometimes used synonymously. This is misleading since there are hybrids that do not exhibit heterosis, but there cannot be

heterosis without hybrids (Lamkey and Edwards 1999). Following Falconer and Mackay (1996), we will define heterosis or hybrid vigour as the difference in performance between the hybrid and the mean of the two parents and call it midparent heterosis.

$$\text{Midparent heterosis (MPH)} = F_I - \bar{P} \text{ with } \bar{P} = \frac{P_i + P_j}{2}$$

This general form can be specified according to the genetic state of the parents (see Lamkey and Edwards 1999).

IMPH: inbred midparent heterosis if the parents are homozygous inbred lines.

PMPH: panmictic-midparent heterosis if two random mating populations are crossed to form an F_1 hybrid, i.e. the difference between the F_1 and the mean of the parent populations. F_2-heterosis is defined as the difference between the mean of the F_2-generation and the midparent value. Random mating in F_1 reduces the F_2-heterosis to 50% of the MPH. The amount of heterosis in a hybrid requires two conditions: (i) directional partial, complete or overdominance at loci controlling the trait of interest and (ii) differing allele frequencies at those loci in the populations or lines to be crossed (Falconer and Mackay 1996). A detailed review of "Quantitative Genetics of Heterosis" was given by Lamkey and Edwards (1999).

In many species *(Dactylis glomerata, Festuca arundinacea* and *F. pratensis, Lolium perenne* and *L. multiflorum* as well as Alfalfa*)* heterosis for yield can be detected (reviews by Kobabe 1983; Brummer 1999, Posselt 2003b). All earlier studies were made under spaced plant conditions. Under these non-competitive circumstances both heterosis and inbreeding depression are much more pronounced (in the order of 50%) than under sward conditions (Foster 1971, 1973; Posselt 1984b, 1989a, b). Breese (1969), who crossed very divergent populations of cocksfoot, stressed the point of adaptation to different environments and showed that the relative amount of heterosis (in this case PMPH) was higher in poorer environments than in high yielding locations. Gaue et al. (2003) also found higher PMPH under low-N as compared to high-N conditions in perennial ryegrass. Compared to other outbreeders, heterosis under sward conditions is rather small (5–20%). This was observed in population hybrids, SI-hybrids as well as CMS-hybrids. However, it should be kept in mind that in all three cases non-inbred or partial inbred parents were crossed and thus not the full amount of heterosis could be observed.

3.2 Identifying Heterotic Patterns

The importance of heterotic groups and patterns has been discussed in detail by Melchinger and Gumber (1998) as well as the interrelationship between genetic diversity and heterosis (Melchinger 1999). If heterotic patterns are not yet known, it is suggested to preselect parents for genetic distance based on molecular or

geographic data, and to produce and test diallel crosses among them. The highest performing cross combinations are used to define heterotic patterns. There is evidence suggesting that adapted populations isolated by time and space are the most promising candidates for heterotic patterns (Melchinger and Gumber1998).

In a study based on molecular genetic distance (GD) among German ecotypes of perennial ryegrass distinct genepools (Northern vs. Southern) were identified (Bolaric et al. 2005), although the association between GD and hybrid performance was rather low (r=0.3). In a further study at Hohenheim, pre-grouping of the genetic materials by geographic distances (Figure 3) leads to a rather high association (r=0.64) between GD and performance of the diallel crosses (Table 11).

Table 11 Annual dry matter yield (ADMY) in Mg ha^{-1} of 28 population crosses (above diagonal), their 8 parent populations (diagonal, in bold); and panmictic midparent heterosis (PMPH) in % (below diagonal, in italics) of perennial ryegrass averaged across 2 years and two locations

P*	1	2	3	4	5	6	7	8	Mean	GCA
1	**13.5**	15.5	14.6	14.4	14.6	14.6	14.2	15.0	14.8	7.2
2	12.9	**14.0**	15.0	13.8	14.2	14.7	14.3	14.0	14.6	4.6
3	10.5	11.7	**12.9**	14.4	13.7	13.2	13.9	14.6	14.2	1.1
4	4.2	−1.8	6.2	**14.1**	13.9	13.4	13.6	13.8	13.9	−2.4
5	8.5	3.5	3.2	0.6	**13.5**	13.0	13.7	13.6	13.8	−3.2
6	11.4	9.7	2.2	0.0	−1.2	**12.8**	13.6	13.6	13.7	−4.3
7	2.5	0.9	2.0	−3.8	−1.5	0.4	**14.3**	13.8	13.9	−2.4
8	9.9	0.7	8.9	−1.3	−0.3	2.2	−1.4	**13.8**	14.0	−0.6

P*: **1** – "Aberavon", **2** – "Fennema", **3** – ecotype PL, **4** – "Weigra", **5**- to **7**– ecotypes D, **8** – ecotype F

Average ADMY of the crosses (14.1 Mg ha^{-1}) was only slightly higher than the mean of the parents (13.6 Mg ha^{-1}). The highest performing cross with 15.5 Mg ha^{-1} was P1 × P2. This combination also shows the highest PMPH (12.9%). These parents originate from Wales and Austria and the geographic distance is therefore rather high. P1 shows positive heterotic effects in all crosses and could be the nucleus of a heterotic group. A second heterotic group, i.e. the "opposite" pool to P1 would be P2. P3 shows high PMPH with both P1 and P2, and the question will be whether a third separate group should be selected. If this is not desirable, then P3 should be combined with P1 because of lower heterotic effects than with P2. P8 has a high yield and shows PMPH in crosses with P1 and P3, but not with P2. Therefore P8 is well suited to be merged with P2. The four parents (1, 2, 3 and 8) showing highest PMPH are also the most distant ones among the whole set of parents tested. All other parents show negative GCA and should not be further considered.

After having identified the two heterotic groups (pool A with P1 and 3 and pool B with P2 and 8) the breeder could either start a hybrid breeding programme as outlined in Figure 12, or broaden the respective gene pools. To assign materials to one of the heterotic groups, two series of testcrosses with the two pools (A and B) as

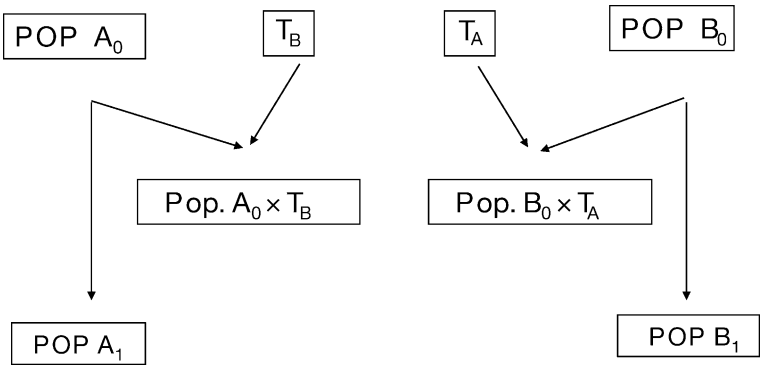

Fig. 12 Generalized scheme of reciprocal recurrent selection for improving two parent populations in hybrid breeding

testers need to be carried out. Populations displaying high PMPH with tester A are assigned to pool B and vice versa. All materials assigned to a particular pool have to be intermated thoroughly to establish the respective base populations.

3.3 Hybrid Breeding

The hybrid breeding scheme in Figure 12 implies that reciprocal testcrosses are made to identify the best genotypes of the candidate hybrids. The best genotypes from each pool are recombined for intrapopulational improvement. In perennial crops the selection units for recombination could be clones or families as previously described. After several cycles of reciprocal recurrent selection when a sufficient yield performance in the testcrosses is reached the best selection units from each pool are the potential hybrid parents. In crops where inbreeding or the production of DH-lines is easy, such as maize, intrapopulation improvement of the respective pools is accompanied by inbred or DH-line development. The final SC is identified by factorial crosses among inbred or DH-lines of the two heterotic groups

3.4 CMS-Hybrids

As an example of CMS-hybrid breeding, hybrid production in rye (*Secale cereale* L.) will be briefly described. Current hybrid varieties are crosses between a CMS-SC as seed parent and a restorer synthetic as pollinator: $(A_{CMS} \times B) \times SYN_{Rf}$. The SC_{CMS} and the SYN derive from different heterotic groups. The hybrid can be classified as a DC, since the pollinator synthetic is based on two inbred lines. Parent B is a self-fertile non-restorer inbred line. In practise, rye hybrids are produced as a mixture of 90% SC_{CMS} and 10% of SYN_{Rf} as pollinator. Thus, the final hybrid consists of about 90% hybrid plants (Geiger and Miedaner 2009).

In grasses with a similar flowering biology hybrid breeding can follow the rye breeding procedure, if inbred lines can be developed. This was done in the former Hohenheim hybrid programme with perennial ryegrass (Posselt 1984): The CMS-source was maintained by crossing to one of the inbred lines available. The BC_2 was used as CMS-tester and the candidates were non inbred plants from the opposite pool (Posselt 1989b). The forementioned BC_2 was crossed to a non-related inbred line to produce the SC_{CMS} as seed parent, while a two-parent synthetic was developed as pollen parent. Hybrid production was like in hybrid rye. The programme had to be abandoned, because the CMS materials were not stable in pollen sterility.

Ruge et al. (2003) reported about a new and stable CMS source (named MSL-CMS) in perennial ryegrass. A practical hybrid breeding programme was initiated and the first hybrids are in VCU trials in Germany (Luesink, NPZ Hohenlieth, Germany; pers. communication).

There are many reports about CMS in other forage crop species (Kobabe 1983, Gallais and Bannerot 1992), however, no CMS-hybrid variety has been listed so far.

3.5 SI-Hybrids

The production of SI-hybrids in grasses based on the gametophytic two-locus incompatibiliy system was proposed by England (1974). The procedure depends on the ability to self an individual genotype to produce the expected portion of genotypes which are homozygous at zero (hom 0), one (hom 1) or both (hom 2) of the loci. Two further generations of intermating the S_1-line by open pollination (R) in isolation are necessary to obtain equilibrium (0.5 hom 0 and 0.5 hom 1) in the S1R2. If two different lines with appropriate SI-genotypes are mixed together, a seed crop is produced which consists maximal (83%) of F_1hybrids. The remaining 17% are inbreds from intermating within the parental S1R2-lines. Under the intense competitive conditions of a sward it is assumed that only the more vigorous hybrid plants will survive (Posselt 1993).

In an experiment 30 S1R2 lines were grouped into three sets with 10 lines each. The 10 lines were crossed in a 5 × 5 factorial manner. The 25 SI-hybrids and the 10 parental lines of each set were field plot tested in comparison to a standard variety at two locations for three successive years. On average, hybrids outyielded the partial inbreds only slightly (7%). The best SI-hybrid yielded 10.3 Mg ha^{-1} ADMY and outyielded the standard variety by about 5%. Highest PMPH was 20% (Posselt 1993). It has to be mentioned though that in this study all inbred lines had been derived from a single gene pool. Additional heterotic effects and thus higher hybrid performance can be expected if the parental lines originate from different heterotic groups.

From the original clones which were used for line development, synthetics were constructed. Twelve SI-hybrids were tested in comparison with their respective Syn-2(2) synthetics. On average the SI-hybrids outyielded the synthetics by 10%.

Eickmeyer (1994) investigated the SI-hybrid system in Italian ryegrass under spaced plant conditions. He found much higher MPH (up to 80%). By means of

electrophoreses he was able to estimate hybridization rates, which often were less than the theoretically expected 83%. Critical points are that the S1R2-lines have to be different in their S- and Z-alleles, time of flowering has to match and equal amounts of pollen should be released by the two lines. A further critical point is the maintenance of the S and Z composition during S_1 multiplication. Migrating S- or Z-alleles are assumed to have selective advantage.

If SI-hybrid production were to be carried out on 100 ha, 2,000 kg of seed would be needed. The 1,000 kg of each line would be produced on 2 ha being isolated as best as possible. The 40 kg for sowing the 2 ha should be produced under pollen-proof conditions in isolation cabinets.

3.6 Semi-hybrids

The general idea of creating population hybrids in grasses has been described by Burton (1948), who suggested the term "chance hybrids". Other authors (Kobabe 1983, Brummer 1999) favour the expression "semi-hybrids". Interpopulational hybridization results in 50% interpopulational and 50% intrapopulational crosses. Theoretically, one-half of the potential heterosis can be exploited in such "semi-hybrids". If the populations derive from distinct heterotic groups, the "semi-hybrid" can display a large proportion of PMPH and also outyield the better parent (Melchinger 1999). An example of the variation in population hybrid performance is shown for perennial ryegrass in Table 11.

In Italian ryegrass, Bertling (1993) analysed parent populations and their offspring populations by means of electrophoresis. In several crosses, the rate of hybridization was much less (35%) than the expected 50%. Flowering synchronization between the two populations is one bottle neck of this system. Occasionally, in diallel population crosses of unrelated materials, significant SCA variance is observed, which is an indication of nonmatching pollination. In many cases, divergent populations will also be different in their phenotypic appearance which may lead to great heterogeneity in the resultant "semi-hybrid" and it is doubtful that DUS requirements can be fulfilled. However, phenotypic similarity can be controlled much easier in narrow populations like full-sib families. Thus, referring to Figure 12, FSF selection is suggested, and the "semi-hybrid" derives from crossing phenotypically similar FSFs.

3.7 Combining Hybrid and Synthetic Breeding

In terms of synthetic breeding, a FSF equals a synthetic of two parents [Syn-1(2)] and reaches equilibrium after further multiplication [Syn-e(2)]. The crossing product of two preselected synthetics [Syn-e(2)A × Syn-e(2)B] is a Syn-1(4)AB or could be called a hybrid. This Syn-1(4)AB should be superior to a four-parent equilibrium synthetic [Syn-e(4)], because in the latter only part of the PMPH is retained.

3.8 GCA vs. SCA

The ratio of σ^2_{SCA}: σ^2_{GCA} is a criterion of the importance of SCA for a particular trait. In maize (Melchinger 1999) it was shown that SCA was much more important for grain yield than for forage yield (12.9 vs. 3.9). No such comparisons are known in the forages. However, it has been assumed that in vegetative traits like biomass production additive gene action is much more important than non-additive effects (Breese and Hayward 1972), while in generative traits like seed yield non-additive effects are more pronounced.

3.9 Further Considerations

In general, hybrids of arable crops are cultivated under monoculture conditions. With the exception of alfalfa, this is not the case in forage crops. They are sown in mixtures of several species. The greater uniformity of hybrids might be of a disadvantage to explore niches in the mixed swards or they might dominate the sward in an unwanted fashion. More probably even, there will be a dilution effect, since only part of the heterosis can be exploited due to the reduced amount of hybrid plants in the mixture. Thus, it is an open question how perennial ryegrass hybrid varieties will perform in association with white clover as a companion crop.

In the amenity grasses, no advantages can be seen for hybrid varieties, since yield performance is not a breeding objective and uniformity is not superior than in synthetics. Moreover, most amenity species are used in mixtures anyway.

4 Breeding Autotetraploids

Gallais (1981) stated, “Fortunately, the general breeding strategy developed for diploids is also valid for autopolyploids.” However, some modifications need to be considered. The interested reader is referred to the textbooks of Wricke and Weber (1986) and Gallais (2003).

4.1 Basics

In autotetraploids we have to discriminate between chromosome and chromatid segregation. The latter is due to the so-called double reduction during meiosis. In most cases chromosome segregation can be assumed.

If a population contains k alleles at one locus, a single tetraploid genotype can carry a maximum of four different alleles and five genotype classes are possible. In natural autotetraploids tri- and tetragenic interactions play an important role while in induced tetraploids digenic interactions are more important (Gallais 2003).

Digenic interactions are different from diploids in that dominance has a broader action. A recessive, negative allele will become homozygous (aaaa) at a genotype frequency of just one-eighth of the allele frequency in an autotetraploid population, compared to one-half the allele frequency in a diploid population. Consequently, plants carrying at least one copy of the dominant, positive allele will be much more prominent in a tetraploid than in a diploid population at the same allele frequency.

4.2 Random Mating

Different from diploids, one-locus equilibrium is not reached after one cycle, due to the gamete-dependent relationship between alleles. While creating a base population from a large number of plants, three generations of random mating will be necessary in tetraploids to obtain a population with a degree of 96.3% of random associations of two alleles. In an equilibrium population genotype, frequencies not only depend on gene frequencies, but also on the type of segregation.

4.3 Inbreeding

Because of the five allelic states, inbreeding does not follow a linear relationship like in diploids. After one generation of selfing, F=1/6 N. Thus, progress towards homozygosity is rather slow (3.8 generations of selfing to reach F=0.5). Contrary to diploids, bi-parental hybridization of tetraploid inbred parents does not completely remove inbreeding effects. Similarly, hybridization of homozygous parents will not reach maximum heterozygosity in the F_1 hybrid. It has been shown (Gallais 2003) that maximum heterozygosity of double crosses is greater than that of single crosses. The expression of heterosis and inbreeding depression in autotetraploids depends on the degree of intra-allelic interactions. Like in diploids, inbreeding depression in S_1 lines under spaced plant conditions is much more pronounced (31%) compared with dense swards (18%) (Gallais 2003)

4.4 Selection

Neglecting epistasis, the genetic variance is composed of four terms: $\sigma^2_G = \sigma^2_A+\sigma^2_D+\sigma^2_T+\sigma^2_Q$, which are the additive, digenic (dominance in diploids), trigenic and quadrigenic genetic variance components. There is experimental evidence that additive variance will be lower than in diploids. This is important to consider for the breeding of induced tetraploid material, while choosing selection at the diploid or tetraploid level. Mansat et al. (1966) postulated that it is more efficient to select on the diploid level before polyploidization to 4x. This is plausible because of the masking of recessive genes by broader dominance action. In quantitative genetic experiments with induced tetraploids, the amount of aneuploids could have severe

effects on means and variances, as shown by Simonsen (1976, 1977) in perennial ryegrass and meadow fescue.

In general prediction formulae for genetic response are the same as for diploids but there is always a contribution from dominance variance. During selection a disequilibrium is generated and partially transmitted by the gametes. This is analogous to the effect of additive × additive epistasis in diploids. In tetraploids, with relaxed selection pressure, 2/3 of σ^2_D from the previous cycle is removed, but a new component is added. This component returns to zero once the population has returned to equilibrium. With only two cycles of random mating, the amount of inbreeding and of panmictic disequilibrium will be reduced by 2/3.

Among-family variances can be described as follows:

$$\sigma^2_{G\,HSF} = \frac{1}{4}\,\sigma^2_A + \frac{1}{36}\,\sigma^2_D$$

$$\sigma^2_{G\,FSF} = \frac{1}{2}\,\sigma^2_A + \frac{2}{9}\,\sigma^2_D \frac{1}{12}\,\sigma^2_T + \frac{1}{36}\,\sigma^2_Q$$

In terms of combining ability variances,

$$\sigma^2_{GCA} = \frac{1}{4}\,\sigma^2_A + \frac{1}{36}\,\sigma^2_D \text{ and } \sigma^2_{SCA} = \frac{1}{6}\,\sigma^2_D + \frac{1}{12}\,\sigma^2_T + \frac{1}{36}\,\sigma^2_Q$$

In practical breeding, the estimates of the nonadditive genetic variances are very small (Dudley et al. 1969) and can be neglected.

In HS-progeny testing it will be better to maintain the mother plants for recombination instead of remnant seed. Like in diploids, σ^2_A is doubled, though σ^2_D is increased in same time.

4.5 Effective Population Size

The number of selected plants in recurrent selection depends largely on the genetic composition and the type of segregation. Like in diploids, the inbreeding coefficient *F* can be used to measure the risk of inbreeding depression. If interactions between alleles are restricted to the first degree (digenic interactions, as in most cases of induced tetraploids), *F* can be calculated by $F_n = 1-(1-1/6\,N)n$, with $F=0$ in the previous generation (n=number of generations). In this case, the size of the population could be three times less than that in diploids for equal *F*. However, with strong interactions between four alleles (tetra-allelic interactions, as can be expected in natural tetraploids like alfalfa; see Chapter17) the number of selected plants should be more than twice as large as that in diploids for the same relative inbreeding effect.

4.6 Synthetic Breeding

For synthetics there is no great difference between diploids and autopolyploids. If interactions between alleles are restricted to the first degree, inbreeding depression will be lower, and for a given F it will be possible to decrease the number of parents. There will be an optimal number of parents at a given level of inbreeding. However, when variances of interactions between more than two alleles are important the optimum number of parents will be greater than for diploids. Formula (3.4) was applied by Reheul (1987) to predict synthetic performance of two series (n=20 and 24 clones) of tetraploid perennial ryegrass. He found an optimum number of parents of six for these two groups of material. In the older literature, which has been reviewed by Becker (1988), the recommended number of parents were 2–9 in *Medicago sativa* and 4–10 in *Dactylis glomerata*. Different to diploids, equilibrium is not reached after one generation of intermating and advanced generations (Syn-3 or Syn-4) should therefore be used for DUS and VCU testing. With homozygous or partially self-fertile parents equilibrium is only reached asymptotically.

4.7 Hybrid Breeding

Single crosses between completely inbred parents result in biallele-duplex types ($B_1B_1B_2B_2$) and thus the coefficient of inbreeding is $F = 1/3$. For double crosses of four unrelated inbred lines it was shown that inbreeding (F=1/9) was less than in single crosses. Though selection among parents is more effective with a high degree of inbreeding this negative effect cannot completely removed in hybrids. Experimental results in alfalfa indicate that two generations of selfing may be the optimum for hybrid parents (Rotili and Zannone 1974). Prediction formulae for double cross and three-way cross hybrids are given by Wricke and Weber (1986). Experimental results on alfalfa showed a slight superiority of double crosses as compared to four inbred parent synthetics.

Establishing two heterotic groups and the development of so-called semihybrids was suggested by Brummer (1999). A scheme for *four-way population improvement* to maximize double cross performance was presented by Gallais (1981). The idea of successive hybridization to further increase heterozygosity and thus exploit progressively heterosis was suggested earlier by Bingham (1980).

5 Application of Molecular and Biotechnological Tools

The term ‘molecular breeding’ is a collective descriptor of the heterogeneous efforts, challenges and opportunities being investigated to enhance the success of the systematic procedures used to improve trait phenotypes by directed manipulation of the genotype at the DNA sequence level. At this time, molecular breeding is not an identifier of a single general breeding approach in the same way that ‘pedigree breeding’ is such an identifier. Thus, many different breeding approaches are considered under the title ‘molecular breeding’.

> Two major components are in use today: i) direct movement of genes between individuals by a range of transgenic approaches, and ii) development of associations between individual DNA sequence variation and trait phenotypic variation in combination with the design of DNA based prognostics that can be used in high throughput systems as a component of a breeding programme. As with most difficult challenges the paths to improvement are many and any commitment to molecular breeding strategy development will be an iterative process. Thus it may be more accurate to refer to 'molecular enhanced' breeding strategies, which apply molecular technologies around what are still predominantly large pedigree breeding strategies (Cooper et al. 2004).

In Section 2 (breeding population varieties), the structuring of breeding methods into breeding phases was introduced. Molecular breeding benefits all three phases, but is also useful in the context of variety registration and protection as well as for the characterization and management of genetic resources (Lübberstedt 2005).

Genetic engineering aims at enlarging the useful genetic variation for the breeder. In most cases, the transgenic approach is only applicable in tissue culture responsive genotypes and needs to be transferred into elite breeding materials. Two procedures for the introgression of new genes or even QTL can be thought of: (i) introduction into the parental clones of already existing varieties (variety-parent approach) or (ii) transfer into a new base population (population approach). In both approaches repeated backcrossing and an efficient selection system are essential. Transgenes must be brought to homozygosity in the variety parents. Introgression of transgenic virus resistance in the white clover breeding programme in Australia applied the variety-parent approach (Smith et al. 2005).

Marker-assisted introgression will basically follow the same rules. The gene or QTL of interest is introduced by crossing and marker-assisted backcrossing (MAB) is applied. Different to the introgression from non-adapted materials no linkage drag is expected if the gene of interest derives from elite breeding material. This is the case in the approach described by Yadav et al. (2003), where high WSC (water soluble carbohydrates) from an elite genotype is combined with crown rust resistance from another elite genotype in a segregating mapping population. These authors intend to select parents for synthetic construction after successful backcrossing to the two parents. Thus, inbreeding will occur and since the potential parents derive from the same FSF, the inbreeding depression is not removed by intermating. Thus, it would be more appropriate to have at least a further parallel programme with genotypes being unrelated to the first FSF. So far, MAS was only successfully applied in the respective mapping population and not in deviating genetic backgrounds. Tightly linked markers are needed to trace favourable alleles or QTL in the so-called foreground selection during backcrossing. With marker-assisted background selection (Frisch and Melchinger 2001) the elite parent background can be recovered efficiently in a shorter time than with conventional procedures.

A further introgression approach was applied by Yamada et al. (2005) in the Lolium/fescue complex. The authors fixed high freezing tolerance in DH-lines as donors for further backcrossing with *L. perenne*. However, they did not consider a complete breeding programme based on the use of DHs. The advantages of DH lines

in recurrent selection were described recently for hybrid maize breeding (Gordillo and Geiger 2008).

DNA markers have been widely used to describe genetic variation in the species of interest, and so far only few attempts have been made to use the diversity information in practical breeding. The relationship between genetic distance (GD) and yield of diallel crosses was investigated in alfalfa (Kidwell et al. 1994) and perennial ryegrass (Posselt 2005). The assumption that genetically diverse parents outyield populations from genetically similar parents was confirmed in alfalfa (Kidwell et al. 1999) and in perennial ryegrass (Kölliker et al. 2005). The latter group selected individual genotypes from a set of elite clones according to their genetic diversity based on AFLP marker data and developed synthetics with contrasting levels of diversity (narrow vs. wide). The more diverse synthetics outyielded the narrow ones.

MAS for QTL of highly polygenic-inherited traits like yield was mostly disappointing and is expected to improve through the application of functional markers (Lübberstedt 2005) or genome-wide selection (Bernardo and Yu 2007).

Yadav et al. (2003) observed patterns of allelic variation for a set of SSRs in perennial ryegrass. On average ecotypes displayed a four times larger number of alleles than breeding populations (19 vs. 5). Allelic variants can also be identified by association mapping (see Chapter 4). High levels of nucleotide diversity were also found in perennial ryegrass natural populations (Skøt et al. 2005). However, to date it is not yet clear if all these allelic variants are useful and furthermore, how their small phenotypic effects can be measured. This leads to the general limitations in phenotyping. Clearly defined and measurable traits, investigated under several environmental conditions, are a prerequisite for tight marker trait associations. Several new technologies like NIRS, FTIR, imaging spectroscopy and others have enormously improved phenotyping. However, many phenotype–genotype associations have been based on the mapped and cloned genotypes per se and under spaced plant conditions. Genome regions increasing GCA for the traits of interest are of highest priority. Hence, evaluation of testcross rather than per se performance and under test conditions close to agronomic practise is requested (Lübberstedt 2005). Markers in combination with quantitative genetic parameters can be employed to predict the performance of synthetic or hybrid varieties using BLUB (Bernardo 2002).

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Development and Application of Biotechnological and Molecular Genetic Tools

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1 Basic Techniques and Technologies

1.1 Cell/Tissue Culture and Production of Doubled Haploids

In vitro culture of plant cells, tissues and organs in forage crops can be aimed at generating haploids or doubled haploids, inducing or selecting for resistance to biotic or abiotic stress, accomplishing somatic hybridisation, embryo rescuing or chromosome doubling of interspecific hybrids and obtaining genetically engineered plants.

Recent papers have thoroughly reviewed these topics in forage grasses and legumes (Samac and Temple 2004, Somers et al. 2003, Wang and Ge 2006).

1.1.1 Haploids and Doubled Haploids

Haploid plants develop from either the male or female gametes without fertilisation and can be obtained in two ways: androgenesis, by anther or microspore culture, or gynogenesis, after pollination with distantly related species (wide crosses) usually followed by *in vitro* rescue of the haploid embryos. Androgenesis is the development of haploids from the male gamete, while gynogenesis (or parthenogenesis) is the development of haploids from the unfertilised egg. Because they arise without fertilisation, or following fertilisation and subsequent paternal chromosome elimination, haploid plants are of either maternal or paternal genotype. Spontaneous or chemically induced chromosome doubling of haploid plants leads to homozygous *doubled haploid* individuals. A haploid individual derived from a diploid plant is a *monoploid*; a haploid derived from a polyploid is a *polyhaploid*; in this latter

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case, the prefix is used to indicate the genetic complement: a *dihaploid* is a haploid obtained from a *tetraploid*, whereas a *trihaploid* is a haploid obtained from a *hexaploid* (Croser et al. 2006).

Androgenesis is conventionally aimed at obtaining haploids from heterozygous diploid plants, and homozygous genotypes by chromosome doubling. However, the most useful outcome of androgenesis in forage grasses has been the generation of populations characterised by high genotypic and phenotypic variation from *Lolium* × *Festuca* amphiploids ($2n = 4x = 28$). These populations displayed abiotic stress resistance higher than that found in the parental genotypes, indicating that the high chromosome recombination within gametes of these hybrids uncovers alleles that, in the disomic state, were hidden by allelic and non-allelic (Humphreys et al. 2007) interactions. This approach can be very useful for introgression breeding and genetic and physiological dissection of complex traits.

A protocol for haploid and doubled haploid induction in forage and turf grasses was published (Andersen 2003) that describes donor plant conditions, anther collection and culture, culture media, growth requirement of the regenerated plants and discusses the efficiency and applications of the method. It is applicable to *Lolium perenne, Lolium multiflorum, Phleum pratense* and *Dactylis glomerata*.

In forage legumes, alfalfa is the only species in which haploids have had an impact on genetics and breeding. Alfalfa dihaploids can be routinely obtained by crossing 4*x* plants by 2*x* pollen donors, exploiting parthenogenesis (reviewed in McCoy and Bingham 1988). The widespread occurrence of parthenogenesis allows obtaining dihaploids from almost any tetraploid alfalfa plant. Dihaploids have been used for studies aimed at elucidating the effect of the number of alleles on vigour in tetraploid alfalfa (reviewed by Bingham et al. 1994). In *Lotus corniculatus*, a dihaploid plant was obtained by pollinating tetraploid *L. corniculatus* with diploid *L. tenuis* pollen (Negri and Veronesi 1989). Haploids via anther culture in forage legumes were reported only in *Medicago sativa*, *L. corniculatus* and *Trifolium alexandrinum* (reviewed by Croser et al. 2006).

The fact that there are very few reports of monoploid plants in forage or turf species indicates that in highly heterozygous, outcrossing plants that likely carry a heavy load of lethal recessive genes, one chromosome set is not enough for survival (Andersen 2003, Croser et al. 2006).

In vitro culture for inducing resistance to biotic or abiotic stress has had limited application in forage crops. Somaclonal variation or genetic variation that arises following *in vitro* regeneration was proposed as a tool to generate useful variation for breeding, but this promise has not been realised (Wang et al. 2001).

In vitro screening, on the contrary, can be useful in breeding programs. For example, callus formation in the presence of toxic aluminium was employed in introgressing acid soil tolerance from diploid to tetraploid *M. sativa* (Sledge et al. 2002).

Embryo rescue is a tool to obtain interspecific hybrids when an endosperm does not develop. Excising and *in vitro* culturing hybrid embryos allowed obtaining hybrids in *Trifolium* (Abberton 2007).

1.1.2 Regeneration and Transformation

Protocols have become available for a number of forage grass and legume species (e.g. Somers et al. 2003, Sullivan and Quesenberry 2006, Wang and Ge 2006). However, *in vitro* regeneration and transformation ability depends on the genotype, and this is a limitation because the highly regenerable and easily transformed genotypes are often unsuitable for direct cultivation. It would be very desirable to be able to transform any genotypes of a variety of choice, thus speeding up considerably the development of biotech varieties.

Genotype-independent transformation protocols have been reported in *Medicago* and other forage legumes (Ding et al. 2003, Weeks et al. 2008). These results should be confirmed in other labs, so that versatile transformation protocols may become generally available. Genotype-independent genetic transformation can be particularly useful in apomictic grasses, in which transformation of elite genotypes may readily translate into improved biotech varieties, thanks to asexual seed formation.

Selectable marker genes are considered to be necessary for efficient transformation of forage plants, and bacterial antibiotic or herbicide resistance genes have been used (Figure 1a). Gabaculine resistance encoded by the bacterial mutant *hemL* gene allowed efficient transformation of alfalfa without antibiotics or herbicides (Rosellini et al. 2007, Figure 1b).

Transformation without selectable markers has been reported in alfalfa (Weeks et al. 2008). Post-transformation marker elimination has not been realised in forage and turf species. It would be useful if alternative markers and marker-free technologies were extended to forage plants.

Transgene expression stability is crucial for the deployment of the transferred traits and must be checked in the sexual progenies of the transformed genotypes (T_0 plants). Expression may decrease in the progenies, but it can also be augmented

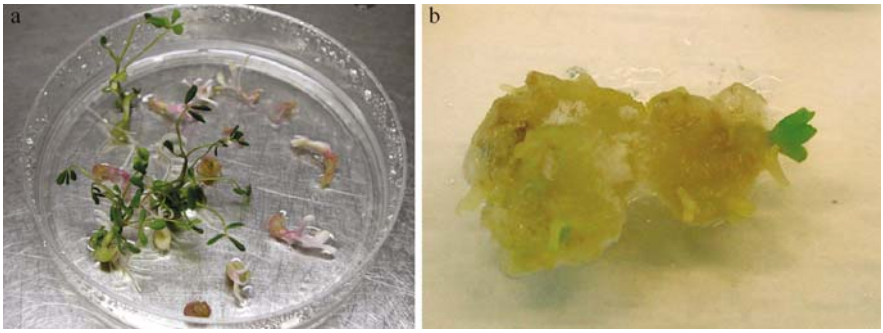

Fig. 1 **a** (*left*): Green alfalfa plantlets developed *in vitro* in the presence of the antibiotic kanamycin, from transgenic somatic embryos expressing the *NptII* gene from *E. coli*, whereas non-transgenic embryos *(pink)* did not develop **b** *(right)*: A transgenic alfalfa somatic embryo obtained in the presence of phytotoxic gabaculine after transformation with the bacterial mutant *hem*L gene (Photo D. Rosellini)

by recurrent selection, as was reported for white clover (*Trifolium repens*; Schmidt et al. 2004).

Reference genes for qPCR have been proposed for alfalfa (Alexander et al. 2007) that can be useful for assessing transgenic contamination levels in forage products and transgene copy number or zygosity.

1.2 Genetic Markers

The concept of using simple "marker" characteristics to select for more complex target traits was first proposed by Sax (1923) who observed an association between pigmentation and seed weight in *Phaseolus vulgaris*. The concept was consequently refined and expanded by many scientists and numerous marker systems targeting phenotypic, biochemical and molecular genetic characteristics have been developed and employed as tools to facilitate selection as well as to analyse genetic diversity in natural and breeding gene pools. In the following, a brief account on the most prominent marker systems is given with emphasis on more recent developments. More details on molecular genetic markers and their application can be found in recent literature (Semagn et al. 2006, Wang et al. 2001).

Isozymes, i.e. multiple molecular forms of an enzyme sharing the same catalytic activity, have been successfully used to analyse genetic diversity in various forage species and they have been proven particularly useful to elucidate genetic relationships in forage grasses such as ryegrasses and fescues (Charmet and Balfourier 1994). Certain isozyme loci have also been found to be linked to traits of agronomic importance. For example, the content of water soluble carbohydrates in ryegrasses has been found to be closely related to specific alleles at the *Pgi2* locus (Hayward et al. 1994). Isozymes are accepted as supplemental discriminating properties in DUS testing. However, the number of isozyme assays is insufficient for most applications in plant breeding and isozyme markers often fail to distinguish between more closely related individuals, limiting their use in surveys of genetic diversity.

Molecular genetic markers are specific fragments of DNA that can be identified within the genome of the organism under study using a broad variety of techniques. The major advantages of molecular markers are their nearly unlimited number and their amenability to automation and high-throughput analysis. Semagn et al. (2006) described more than 30 different marker types which may be classified according to their mode of transmission (biparental, paternal or maternal inheritance), their mode of expression (dominant or co-dominant), the method of detection (PCR based or hybridisation based) or their location in the genome (random- or targeting-specific regions). However, for applications in forage crops which comprise many different, often genetically poorly characterised species, a distinction between markers which require no a priori sequence information (e.g. RAPD, AFLP or ISSR markers; see below) and markers which are based on specific DNA sequences (e.g. RFLP, SSR or SNP markers; see below) may be more appropriate.

RFLP – restriction fragment length polymorphism markers are highly reproducible, can be scored co-dominantly and may be conserved across species and genera. RFLP probe libraries have been developed specifically for forage species such as tall fescue (*Festuca arundinacea*; Xu et al. 1991) or ryegrass (Hayward et al. 1998), but the number of markers has been augmented in many studies by the use of probes from species such as rice or wheat (Jones et al. 2002). Although RFLP markers have been used for the analysis of genetic diversity or the construction of linkage maps in various forage species, their extensive use has always been hampered by high-cost and labour requirements, and the general lack of sequence information for many forage species.

RAPD – randomly amplified polymorphic DNA markers do not require a priori sequence information and a large number of markers can be generated in a short time. This is why RAPDs rapidly became the markers of choice for many forage species where no DNA sequence information was available such as red clover (Kongkiatngam et al. 1995) or meadow fescue (Kölliker et al. 1999). Despite their advantages, RAPD markers can only be scored dominantly for marker presence (i.e. heterozygous individuals cannot be distinguished from homozygous individuals), they are often not transferable across different populations and suffer from reproducibility problems.

AFLP – amplified fragment length polymorphism are also anonymous DNA markers. They are based on a more sophisticated technique of detection and are characterised by high reproducibility and better transferability when compared to RAPD markers. Their reliability and suitability for high-sample throughput have made AFLP markers very popular for the analysis of poorly characterised plant species. In forage crops, AFLP markers have been widely used for the analysis of genetic diversity as well as for QTL and linkage analysis in forage species (e.g. Herrmann et al. 2006, Skot et al. 2002). Since co-dominant scoring of AFLP markers is difficult, they are predominantly treated as dominant markers.

SSR – simple sequence repeat markers are based on PCR amplification of short repeated sequence motifs of two to six nucleotides, which are ubiquitous in eukaryotic genomes. Their ability to detect polymorphism between closely related individuals and their co-dominant nature together with their sequence specificity and high reproducibility make SSR markers invaluable tools for genetic dissection of agronomic traits and analysis of genetic diversity in a broad range of species including ryegrasses (Jones et al. 2001) and clovers (Sato et al. 2005). Probably the only drawback of SSR markers is their laborious development which mostly involves construction and sequencing of genomic and/or cDNA libraries.

SNP – single nucleotide polymorphism markers are based on single base pair changes in a DNA sequence and have so far only been reported for two forage species, perennial ryegrass (Cogan et al. 2006) and white clover (Cogan et al. 2007). In perennial ryegrass, marker-trait associations were found for disease resistance (Dracatos et al. 2008) and water-soluble carbohydrate content (Skot et al. 2007). Due to their great abundance and the rapid methodological advances for high-throughput development and detection, SNP markers will no doubt play an important role in the future of marker-assisted forage crop breeding.

DArT – diversity array technology is a microarray hybridisation-based technique which allows simultaneous detection of thousands of markers without a priori knowledge of DNA sequence (Jaccoud et al. 2001). However, interesting markers may be sequenced a posteriori, adding further value to the analysis. So far only one report on the development and use of DArT in forage crops is available for ryegrass and fescue species (Kopecky et al. 2009).

1.3 DNA Sequencing

The publication of the dideoxy sequencing method by Sanger et al. (1977) marked the start of decades of sequencing-driven research in plant sciences and a number of model genomes such as the ones of *Arabidopsis* or rice were sequenced largely based on this method. However, genome sequencing remained an expensive and laborious undertaking and was not applicable to species with large genomes such as wheat, barley or ryegrass (Stein 2007).

Next-generation sequencing technologies, commercially available since 2005, offer a dramatic increase in cost-effective sequence throughput and have been rapidly adopted by the scientific community. A thorough review of the most important technologies such as 454 pyrosequencing (Roche Applied Sciences), the Illumina/Solexa approach (Illumina Inc.) and the supported oligonucleotide ligation and detection system SOLiD (Applied Biosystems) was given by Morozova and Marra (2008). All three technologies are capable of generating several Gb of sequence in a few days making also large genomes accessible. However, these techniques normally produce sequences of short length, making the assembly of sequences particularly demanding (Stein 2007).

Up to now, no complete genome sequence of a forage grass or legume species has been published. However, genomes of model species such as rice or *Arabidopsis* may be useful through comparative genetic approaches (see below). In addition, thanks to the rapid development of more affordable sequencing technologies, genome information of a number of forage species or closely related relatives such as *Medicago truncatula*, *Lotus japonicus* or *Brachypodium distachyon* will become publicly available in the near future (http://www.genomesonline.org/). Despite the lack of complete genome information, quite a substantial amount of sequence data has been published for various forage species. These resources include large collection of expressed sequence tags (ESTs) for species such as perennial ryegrass (Sawbridge et al. 2003b), tall fescue (Mian et al. 2008), white clover (Sawbridge et al. 2003a) and red clover (Sato et al. 2005), and sequences associated with specific functions such as resistance gene analogues or sequence-associated molecular markers (Cogan et al. 2006).

2 Analysis and Utilisation of Genetic Diversity

Genetic diversity within species, i.e. the diversity among individuals within populations is not only the prerequisite for successful population improvement based on artificial selection, it is also important for optimal performance of populations

in a broad range of environments. This may be particularly important for cultivars of forage and turf species which are often used for several purposes in different environments.

2.1 Methodological Considerations

Molecular markers allow for a rapid assessment of genetic diversity directly at the genome level. The choice of the marker system employed depends on the aim of the study and the availability of sequence-specific markers for the species of interest. Due to the initial lack of genetic information, anonymous marker systems such as RAPD, ISSR and AFLP have long dominated research on genetic diversity of forage and turf species (Kongkiatngam et al. 1995, Touil et al. 2008). These methods yield multi-locus genetic data in a single PCR assay and are therefore highly cost-effective. Improved detection techniques have resulted in high reproducibility and comparability of some of these methods. Thanks to numerous efforts, co-dominant SSR markers have become publicly available for several forage and turf species (e.g. Jones et al. 2001, Sato et al. 2005). In studies where a distinction between homozygous and heterozygous individuals is not mandatory, multiplex markers such as AFLP or DArT may be more cost-effective while SSRs certainly have advantages in trait dissection studies. However, the variability detected with the markers mentioned above is often poorly correlated with the variability of traits relevant for survival and performance. Functional markers directly linked to specific traits or functions (Andersen and Lübberstedt 2003) would allow for a more targeted characterisation of genetic resources. So far, only a few gene-associated markers have been reported for forage and turf species (e.g. Cogan et al. 2006, Miura et al. 2007) but the rapid advances in genomic technologies will certainly allow for progress in this area.

The outbreeding reproduction system of many forage species requires a substantial number of genotypes to be sampled per population to characterise an adequate proportion of the genetic diversity present. For example, to sample genotypes which occur at a frequency of 0.01 with a probability of 0.95, approximately 300 plants have to be sampled (Crossa 1989). Practical reasons often prohibit such an extensive sampling and sample sizes as small as 10 or 20 are frequently found in the literature. Although a loss of very rare alleles may even be desirable for studies focussing on distinction of populations, a sample size of at least 40 individual plants, which allows genotypes with a frequency of 0.05 to be detected at a probability of 0.95, appears appropriate for most investigations (Crossa 1989, Herrmann et al. 2005).

The techniques available allow generation of large data sets of information on many genetic loci in a large number of individuals, but biologically meaningful and statistically sound conclusions are sometimes difficult to extract. In studies on different species, sampling sites and/or populations, analysis of molecular variance (AMOVA, Excoffier et al. 1992) offers a powerful means to partition the variance observed according to its sources (Table 1). Multivariate techniques such

Table 1 Analysis of molecular variance (AMOVA) for 8 landraces and 11 cultivars of red clover using 276 polymorphic AFLP markers (Kölliker et al. 2003, © Springer 2003)

	df	Sum of squares	Variance component[a]	% of total variance
Variance among groups[b]	2	1282.0	4.0	12.0
Variance among landraces and cultivars	16	1451.0	2.7	8.1
Variance within landraces and cultivars	431	11,454.7	26.6	79.9

[a]Components were significant at $P < 0.001$; the probability of obtaining a more extreme random value computed from non-parametric procedures (1000 data permutations).
[b]Three groups consisting of eight Mattenklee landraces, eight Mattenklee cultivars and three field clover cultivars, respectively

as principal component analysis (PCA), discriminant analysis, cluster analysis or model-based clustering methods allow examination of relationships among individuals or inference of population structure. An extensive description of statistical methods can be found in specialised reviews (e.g. Bonin et al. 2007, Mohammadi and Prasanna 2003). However, multivariate techniques are purely descriptive and hypotheses have to be tested wherever possible. For example, bootstrapping may be used to estimate the statistical support to nodes in cluster analysis or redundancy analysis may be applied to relate a set of dependent variables (i.e. molecular marker data) to a set of independent variables (i.e. environmental factors, Hartmann et al. 2005).

2.2 Applications in Forage Crop Breeding

Characterisation of germplasm collections has received a lot of attention due to its importance for plant breeding. Breeding germplasm and natural populations have been analysed in a large number of species including clovers, ryegrasses and fescues (George et al. 2006, Kölliker et al. 1999). Such studies not only yielded valuable information on the geographical distribution of genetic diversity, they may also offer insight into evolutionary and ecological processes important for the long-term management of genetic resources. For example, studies on red clover showed that Mattenklee, a distinct Swiss form of red clover, originated from germplasm introduced from Flanders and Brabant and was not directly related to indigenous populations of wild red clover (Herrmann et al. 2005, Kölliker et al. 2003). SSR analysis within meadow fescue showed a distinct influence of habitat and management on the genetic diversity of ecotype populations. Thus, for preserving a large amount of genetic diversity, sampling from many distinctly different habitats was recommended for this species (Peter-Schmid et al. 2008).

Parental selection may also be supported by knowledge of genetic diversity. This would be particularly useful in outbreeding forage grass species where breeding

often relies on intercrossing several selected parents using the polycross method. Marker-assisted parental selection may allow optimisation of parental combinations in order to maximise heterosis and to minimise inbreeding. For example, a study in perennial ryegrass showed that selection of genetically diverse parents led to better agronomic performance in first and second generation progenies (Kölliker et al. 2005), but many studies failed to show a direct correlation between molecular marker diversity and heterosis (Riday et al. 2003).

Ex situ *conservation of plant genetic resources* is important for maintaining a diverse gene pool but the utilisation of gene bank material is often hindered by the large size and heterogeneous structure of many collections. Molecular markers may provide an additional tool to establish core collections which largely represent the genetic diversity of a crop species.

3 Molecular Dissection of Target Traits

Insights into the genetic control of complex target traits, the ultimate prerequisite for the application of molecular genetic tools in plant breeding, may be gained by directly investigating the target species or by deducing genetic information from other species using comparative genomic approaches.

3.1 Characterisation of Quantitative Trait Loci (QTL)

Most target traits of particular importance in forage crops such as dry matter yield, forage quality or stress resistance show continuous variation due to the joint segregation of several genes, generally referred to as quantitative trait loci (QTL) which individually have a small effect on the phenotype. Due to the advent of molecular markers in the late 1980s it is now possible to obtain approximate locations for QTL, estimate their phenotypic effect and make them accessible to plant breeders through marker-assisted breeding (Kearsey and Luo 2003).

3.1.1 Linkage Mapping

A detailed genetic linkage map is the backbone of any study on QTL identification since it allows locating QTL in relation to molecular marker positions. Genetic linkage maps are constructed by genotyping segregating mapping populations and calculating recombination frequencies among parents and offspring. The genetically most easily accessible types of mapping populations such as F_2 populations, recombinant inbred lines or backcross populations are often difficult to produce for most forage crop species because their high degree of self-incompatibility prevents the development of inbred lines. Thus, crosses between two heterozygous individuals resulting in two-way pseudo-testcross populations are often used in

self-incompatible species such as Italian ryegrass (Studer et al. 2006) or red clover (Herrmann et al. 2006).

The first linkage maps for forage crops were published for diploid alfalfa and an interspecific cross between perennial and Italian ryegrass, mainly based on RFLP markers (Brummer et al. 1993, Hayward et al. 1994). Aided by the development of high-throughput molecular markers, existing maps have been refined subsequently and additional maps have been constructed for many species such as meadow fescue, tall fescue, Kentucky bluegrass and white clover (reviewed in Inoue et al. 2007). In addition, for Italian ryegrass and red clover, high density linkage maps consisting of more than one thousand markers have been made available (Inoue et al. 2004, Sato et al. 2005).

In order to locate and use QTL and candidate genes of interest across different populations, consensus linkage maps that combine information from multiple mapping populations have been established for many crop species. Although the ryegrass map initially published by Hayward et al. (1994) has often served as a reference, limited availability of transferable markers has prevented the development of consensus maps for many forage species. However, recent efforts produced the first consensus linkage map for red clover based on 1804 marker loci and 6 mapping populations (Isobe et al. 2009, Figure 2).

3.1.2 QTL Analysis

QTL are identified by correlating the trait phenotype with the marker genotype taking into account the genetic structure of the mapping population used (Kearsey and Luo 2003). One of the most powerful methods for modelling QTL in segregating populations is commonly known as "interval mapping". It systematically searches all possible QTL locations within every chromosomal interval flanked by a pair of adjacent marker loci. The ratio of the likelihood there being a QTL at a particular location and the likelihood there not being a QTL is calculated and converted into the logarithm of odds (LOD) score which is asymptotically distributed as a chi-square distribution (Kearsey and Luo 2003). Population size substantially influences accuracy and power of QTL analyses. Populations consisting of 100 or fewer genotypes only allow to detect QTL with very large effects (Charmet 2000). Intermediate populations of 200–400 genotypes allow to estimate QTL with better accuracy, but increasing population size to 1000 or more still substantially improves QTL estimation (Schön et al. 2004).

In forage crop species, QTL for a broad variety of traits have been identified which provide the foundation for gene targeting, isolation and marker-assisted selection. Traits investigated include forage quality traits, vernalisation response, heading date in perennial ryegrass, lodging resistance in Italian ryegrass, and yield, winter hardiness and growth characteristics in alfalfa (reviewed in Inoue et al. 2007). Due to its economic importance, disease resistance in forage and turf grasses has gained particular attention. Various QTL for resistance to diseases such as crown rust, bacterial wilt or dollar leaf spot have been identified (Bonos et al. 2006, Dracatos et al.

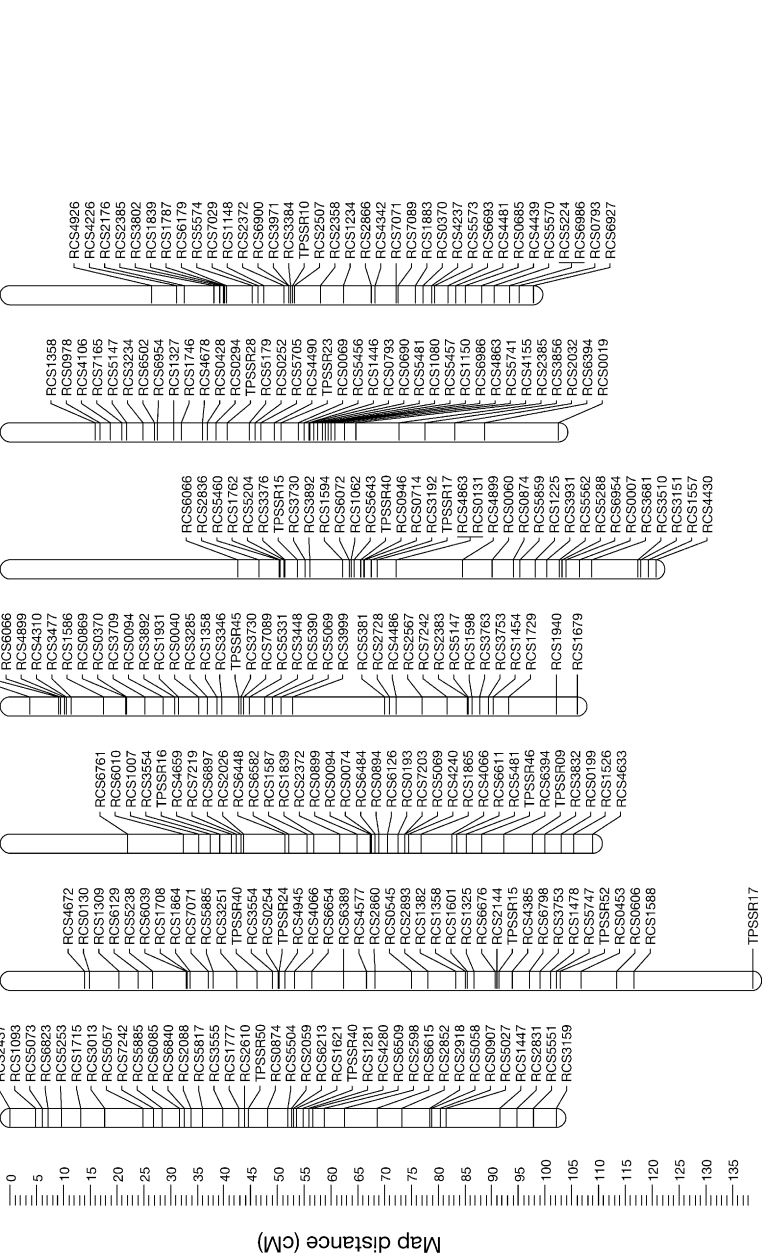

Fig. 2 Consensus linkage map for red clover based on 1804 marker loci and 6 mapping populations (Isobe et al. 2009). For better readability, only markers which bridge individual linkage maps (consensus markers) are show

2008, Studer et al. 2007, Studer et al. 2006). In addition, QTL analysis may also be used to elucidate physiological processes such as the role of fructan in growth and drought response in perennial ryegrass (Turner et al. 2008).

3.1.3 Expression Profiling

The identification of candidate genes, possibly involved in controlling the trait of interest may substantially support QTL analysis and may facilitate the development of markers for MAS. One possibility for identifying candidate genes is the analysis of changes in gene expression profiles under conditions relevant for the trait of interest. cDNA–AFLP analysis allows for the detection of differentially expressed transcripts without the need of sequence information for the species investigated and has been successfully used to identify transcripts differentially expressed in Italian ryegrass during infection with bacterial wilt (Rechsteiner et al. 2006) or to identify key components in the self-incompatibility response of perennial ryegrass (Van Daele et al. 2008). Since the sequencing of differentially expressed transcripts identified through cDNA–AFLP is very laborious, usually only a few candidate genes were identified in these studies.

In microarray analysis, gene-associated DNA is immobilised onto a solid array (microarray) to which target cDNA (or cRNA) is hybridised. Gene expression is usually visualised and quantified by fluorescence-based detection of labelled targets. Due to the large number of short DNA sequences present on an array, expression of thousands of genes can be analysed in a single hybridisation reaction. Microarray platforms have been developed for many crop species such as rice, wheat and maize and a large amount of expression data is available through public databases. So far, no microarray platforms for forage and turf species have been made publicly available. Microarray analyses have therefore been performed using arrays developed for model legume and grass species such as *M. truncatula* (Chandran et al. 2008) or *Festuca mairei* (Wang and Bughrara 2007). However, Ciannamea et al. (2006) used a *L. perenne* cDNA microarray to identify genes upregulated during vernalisation in perennial ryegrass with high homology to members of MADS box, CONSTANS-like and JUMONJI families which are known to play a role in vernalisation of Arabidopsis.

3.2 Comparative Genetics and Genomics

Comparative genetics and genomics is the analysis and comparison of genes and genomes of different species. Such comparison allows for a better understanding of function and location of genes as well as how species have evolved. Because homologous genes are often located in similar genome locations and their function is similar in different plant species, model species offer promising opportunities for comparative genetics and genomics studies.

3.2.1 Model Species

Arabidopsis is a well-known model plant with its genome sequence completed in 2000. The combination of a sequenced genome and the availability of a range of genetic resources such as defined ecotypes or mutant populations derived from chemical or DNA-based (insertion or deletion) mutagenesis greatly facilitates the use of Arabidopsis for gene discovery and annotation (Dixon et al. 2007). However, comparative genome analysis between the model legume *M. truncatula* and Arabidopsis reveals a lack of extensive macrosynteny between these two genomes; it is evident that Arabidopsis cannot serve as a specific model for the structure of legume genomes (Zhu et al. 2003). In forage legumes, *Medicago truncatula*, a close relative of alfalfa, and *L. japonicus*, a leguminous weed, were selected as model species, well suited for studying biological issues important to related forage and crop legume species, such as nitrogen fixation (Young et al. 2005).

Rice (*Oryza sativa*) has been successfully developed as a model system for monocot species. Although information obtained from rice can be useful for forage and turf grasses, these have unique features and traits for their improvement are often different from those of major cereal crops (Wang et al. 2005). The small grass *B. distachyon* (purple false brome) is being developed as a model species because it is more closely related to cool-season grasses that grow in temperate environments than is rice (Garvin 2007). Another grass species, *Lolium temulentum* (Darnel ryegrass), has been proposed as a model plant for genetic manipulation studies in forage and turf grasses (Wang et al. 2005). It has been shown that *L. temulentum* is very closely related to the *Festuca–Lolium* complex as well as other important grasses (Mian et al. 2005). When compared with other forage or turf grass species, *L. temulentum* has the following advantages: autogamous, diploid, no vernalisation requirement, short life cycle and extremely easy to grow in the greenhouse. An efficient transformation system has been developed for *L. temulentum* (Ge et al. 2007).

3.2.2 Synteny

Shared synteny describes preserved co-localisation of genetic loci on chromosomes of related species; it is very useful for establishing the orthology of genomic regions in different species. Comparative genetic mapping has revealed a high degree of conservation in genome structure among closely related plant species, in terms of gene content, order and function (Zhu et al. 2003). Orthologous markers transferable between distantly related species allow for the rapid generation of genetic maps in species where there is little pre-existing genomic or EST information. The comparative genetic maps and syntenic information can then be used to identify markers that are tightly linked to the genes of interest, candidate gene(s) for a trait, and expedite the isolation of such genes (Phan et al. 2006).

By taking advantage of abundant EST sequence information from *M. truncatula*, cross-species genetic markers were developed, and locus orthology was tested through phylogenetic analysis. As expected, the degree of synteny is correlated

with the phylogenetic distance of legume species. The genomes of *M. truncatula* and alfalfa share highly conserved nucleotide sequences and exhibit nearly perfect synteny (Zhu et al. 2005). Synteny analysis in *L. japonicus* detected traces of whole-genome duplication and the presence of synteny blocks with other plant genomes to various degrees (Sato et al. 2008). The conserved genome structure between *M. truncatula* and other legumes has allowed for map-based cloning of genes of interest. One example is a nodulation receptor kinase gene that is required for both bacterial and fungal symbiosis. Map-based cloning and a complementation test were performed in *M. truncatula* and eventually led to the simultaneous cloning of three orthologous genes in *M. truncatula*, alfalfa and pea (Zhu et al. 2005).

A remarkable conservation of gene content and order has been established in comparative genetic mapping experiments for the Poaceae family, although genome sizes vary as much as 40-fold between some of the species, and despite the fact that they diverged as long as 60 million years ago (Schmidt 2000). Given a high degree of genome collinearity at a broader genetic level as well as at the gene level, comparative genome mapping experiments can serve as an efficient tool for transferring information and resources from well-studied genomes to related plants. Comparative mapping identified a region of synteny between rice and perennial ryegrass, which contains the Hd3 heading-date QTL in rice and a major QTL accounting for up to 70% of the variance associated with heading date, in perennial ryegrass. The identification of synteny between rice and perennial ryegrass in this region demonstrates the applicability of the rice genome to the understanding of biological processes in other species (Armstead et al. 2004). Based on comparative genetic studies, synteny for *CBF* gene family was observed between the Triticeae cereals and perennial ryegrass (Tamura and Yamada 2007).

4 Expanding Genetic Variation

Biotechnology and molecular biology offer a variety of tools to enlarge genetic variation and introduce novel genes or alleles into plant breeding programs. A few of the methodologies available are described below. An overview on how to employ these technologies in plant breeding programs to produce improved elite cultivars is given in Chapter 3 of this volume (Section 5).

4.1 Interspecific Hybridisation

Although interspecific hybridisation does not necessarily make use of modern biotechnological tools it has the potential to exploit variation across the sexual compatibility barriers and is a tool to understand evolutionary relationships. Its most useful application is the introgression of genes and traits between species, allowing broadening of the gene pool available to breeders. Interspecific hybridisation can be obtained either by sexual crossing or by *in vitro* fusion between somatic cells and subsequent regeneration of plants from the hybrid cells. To date, only the former has given practical outcomes namely in the creation of intergeneric hybrids in *Festuca*

and *Lolium* or *Medicago* and *Trifolium* which are described in detail in Chapters 12 (*Festulolium*) and 17 (alfalfa).

4.2 Marker-Assisted Selection (MAS)

Molecular markers closely linked to genes or QTL controlling target traits may be used to select for superior genotypes or to introgress novel genes or alleles into breeding germplasm. This may be particularly attractive for traits with phenotypes which are difficult or laborious to determine, such as water soluble carbohydrate content (Wilkins and Humphreys 2003) or seed yield (Herrmann et al. 2006). If appropriate markers are available, MAS may even allow for the simultaneous selection of several unrelated traits in a single step (Van Berloo and Stam 2001). In wheat, MAS has been shown to provide economic advantage when expensive field experiments can be replaced by relatively inexpensive marker analyses (Kuchel et al. 2005). The availability of very tightly linked molecular markers is a prerequisite for application of MAS in genetic backgrounds other than the initial mapping populations. Due to this limitation, there are only few examples of MAS in forage crop species (Humphreys 2005). However, Barrett and colleagues (2009) demonstrated the potential of MAS for improving seed yield in white clover. Using SSR markers linked to a seed yield QTL of moderate resolution they analysed marker-trait associations in 12 breeding pools. Diagnostic SSR markers explained an average of 38% of the difference in seed yield indicating the value that can be realised from MAS in white clover.

Molecular markers may also be used to introgress several resistance genes or QTL into single genotypes or cultivars. With the large number of resistance genes and QTL being discovered, this may also be feasible in forage crop species, at least ryegrasses. However, the methodological considerations for introgression in outbreeding species described in Chapter 3 have to be taken into account and combined efforts have to be undertaken to develop successful strategies for the introgression of multiple disease resistance in forage cultivars.

4.3 Genetic Engineering

4.3.1 Grasses

The first transgenic grass plants were obtained by direct gene transfer to protoplasts (Wang et al. 1992), but the technique is fairly complicated and microprojectile bombardment (biolistics) and *Agrobacterium* transformation have become the main methods for producing transgenic grasses.

The biolistic method was developed as a necessity to transform species initially considered recalcitrant to *Agrobacterium* transformation. Biolistic transformation has led to the successful production of transgenic plants in many forage and turf grasses, including creeping bentgrass, tall fescue, red fescue, perennial ryegrass,

Italian ryegrass, wimmera grass, orchardgrass, Kentucky bluegrass and switchgrass (Wang and Ge 2006). Although biolistic transformation involves a physical process and thus is a fairly reproducible procedure, it tends to produce transgenics with multiple copy integrations of the transgenes.

In recent years, significant progress has been made in developing transformation protocols in grasses based on the use of *Agrobacterium tumefaciens*. Transgenics have been obtained by *Agrobacterium*-mediated transformation in creeping bentgrass, tall fescue, perennial ryegrass, Italian ryegrass, colonial bentgrass, zoysiagrass, and orchardgrass (Wang and Ge 2006).

Transgenic technologies have been used to improve agronomic characteristics of grasses, such as forage quality, stress tolerance and development of hypo-allergic grasses. Feeding and grazing studies with conventionally bred cultivars have shown that small changes in forage digestibility can have a significant impact on animal performance (Casler and Vogel 1999). Lignification of plant cell walls has been identified as the major factor limiting digestibility of fodder crops. The development of antisense and RNAi technologies allows the down-regulation of endogenous genes. In tall fescue, genes encoding major lignin biosynthetic enzymes, cinnamyl alcohol dehydrogenase (CAD) and caffeic acid *O*-methyltransferase (COMT), were cloned and characterised. Down-regulation of CAD and COMT in transgenic tall fescue led to reduced lignin content, altered lignin composition and significantly increased *in vitro* dry matter digestibility (Chen et al. 2004). Lignin also negatively affects the utilisation of plant structural polysaccharides for ethanol production. Therefore, reducing lignin content will also reduce recalcitrance to saccharification in cellulosic bioenergy crops such as switchgrass.

Another interesting utilisation of transgenic technology is the development of hypo-allergic grasses. Grass pollen is a widespread source of airborne allergens and is a major cause of hay fever and seasonal allergic asthma. Genes encoding major allergenic proteins (e.g. Lol p 1, Lol p 2) were cloned from ryegrass. The introduction of antisense *Lol p 1* and *Lol p 2* transgenes into ryegrass plants led to a reduction in accumulation levels of corresponding allergens in pollen (Petrovska et al. 2004). The development of hypo-allergenic grass cultivars may have some potential to benefit public health.

Transgenic perennial ryegrass plants were produced with the wheat fructosyltransferase genes, *wft1* and *wft2*, which encode sucrose–fructan 6-fructosyltransferase (6-SFT) and sucrose–sucrose 1-fructosyltransferase (1-SST; Hisano et al. 2004). Significant increases in both fructan content and freezing tolerance at the cellular level were detected in the transgenics (Hisano et al. 2004). The transgenic expression of isopentenyl transferase (IPT) gene in tall fescue also led to improved cold tolerance (Hu et al. 2005). Transgenic perennial ryegrass carrying a vacuolar membrane Na^+/H^+ antiporter gene displayed significantly enhanced salt tolerance (Wu et al. 2005). Inhibition of floral development was achieved in red fescue by transgenically expressing a strong floral repressor *TERMINAL FLOWER1* (*LpTFL1*) (Jensen et al. 2004). The ability to manipulate flowering is important for limiting pollen-mediated transgene flow (Jensen et al. 2004, Wang and Ge 2006).

4.3.2 Legumes

Among the forage legumes, alfalfa is by far the species to which genetic engineering has been applied most extensively. The first biotech forage variety to be released, in 2006, was Roundup Ready alfalfa (see later). Virus-resistant white clover biotech varieties are also close to marketing (Smith et al. 2005).

The scope and accomplishments of alfalfa transformation have been reviewed earlier (Rosellini and Veronesi 2002, Samac and Temple 2004). Here, the most important traits recently introduced by genetic engineering in forage legumes are briefly reported.

Plant performance in alfalfa, overexpression of a soybean cytosolic glutamine synthetase was realised and resulted in slight increases in total protein content and photosynthetic rates (Seger et al. 2009).

Herbicide tolerance glyphosate tolerance of alfalfa was attained by expression of the *A. tumefaciens* CP4 EPSPS gene and backcrossed into a synthetic variety (reviewed in Samac and Temple 2004). Coexistence guidelines for an insect-pollinated legume crop were developed for the first time for glyphosate tolerant alfalfa (CAST 2008). Atrazine tolerance was introduced into alfalfa by expressing a modified bacterial atrazine chlorohydrolase gene. It is suggested that atrazine-degrading alfalfa may be useful for remediation of atrazine-impacted environments (Wang et al. 2005).

Tolerance to acid soils (or to Al^{3+}) has been pursued with biotech tools by organic acid overproduction in the root tips through overexpression of alfalfa malate dehydrogenase (Tesfaye et al. 2001) or *Pseudomonas aeruginosa* citrate synthase (Barone et al. 2008). In white clover, Al^{3+} tolerance is being pursued with a similar strategy (Mouradov et al. 2007).

Improved phosphorus (P) nutrition could be realised by expressing enzymes that hydrolise phosphate from soil organic compounds such as inositol phosphate (or its cation-associated derivative, phytate). Recently, *M. truncatula* purple acid phosphatase and fungal phytase genes were expressed in white clover (Ma et al. 2009) and demonstrated to be able to markedly improve *P* assimilation when phytate was the only source of *P*. Improved *P* nutrition in the field remains to be demonstrated.

Delay of leaf senescence was obtained in alfalfa by expressing a bacterial *ipt* gene under the control of the *Arabidopsis* leaf senescence-specific SAG12 promoter (Calderini et al. 2007). The usefulness of this stay-green trait in a crop that is clipped at the onset of flowering should be tested.

Virus tolerance was successfully introduced in *T. repens* by expression of the alfalfa mosaic virus (AMV) coat protein gene, and an improved variety is under field testing towards commercial release (Godfree et al. 2006, Mouradov et al. 2007).

Insect resistance a soybean trypsin inhibitor was expressed in leaves of white clover, but further studies are required to define the usefulness of this strategy for insect resistance (Mc Manus et al. 2005).

Drought stress tolerance overexpression of WXP1, a putative AP2 domain-containing transcription factor gene from *M. truncatula* was associated with reduced water loss and chlorophyll leaching from leaves in alfalfa. Enhanced drought

tolerance was demonstrated by delayed wilting under drought stress and quicker and better recovery after re-watering (Zhang et al. 2005).

Forage protein quality increasing the level of sulphur or essential amino acids to improve protein quality of alfalfa has been proven difficult (e.g. Galili et al. 2000, Holguin et al. 2008). However, a recombinant protein between phaseolin and gamma zein did accumulate to high concentration in the endoplasmic reticulum (Bellucci et al. 2007).

Introducing proanthocyanidins (condensed tannins) into alfalfa has long been pursued with the aim to ameliorate protein digestibility and reduce bloating in ruminants. Expression of a maize anthocyanidin regulatory gene (Lc) produced proanthocyanidins and anthocyanidins but modest decrease of initial rate of protein degradation in the rumen (Ray et al. 2003, Wang et al. 2006); the authors concluded that higher concentrations of condensed tannins are required.

Proteolytic loss occurs when some forages are preserved by ensiling. In alfalfa, these losses amount to 44–87%; in contrast, red clover has up to 90% less proteolysis during ensiling, thanks to the combination of polyphenol oxidase (PPO) activity and the presence of *o*-diphenol PPO substrates. Alfalfa expressing a red clover PPO gene had fivefold decrease in proteolysis when a PPO substrate was added (Sullivan and Hatfield 2006)

Forage digestibility Antisense down-regulation of lignin biosynthesis genes such as caffeic acid 3-*O*-methyltransferase (COMT) and caffeoyl CoA 3-*O*-methyltransferase (CCOMT) resulted in improved dry matter digestibility of alfalfa in lambs (Mertens et al. 2008).

Antisense silencing of the lignin biosynthesis enzyme hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase in vascular tissues of alfalfa dramatically changed lignin composition (Shadle et al. 2007) and the most severely down-regulated lines exhibited stunting, reduction of biomass and delayed flowering. Reduced lignin levels might also constitute a transgene containment tool (genetic mitigation), since the reduced fitness of low-lignin plants limits their invasiveness outside pure stands (Weeks et al. 2008).

High value proteins such as industrial enzymes, biodegradable plastics, antigens and antibodies may be produced in forage legumes (Floss et al. 2007). Antigens from various pathogens were generally capable of inducing immune responses when expressed in transgenic alfalfa or white clover, showing that this may be a valuable strategy for vaccine production. However, gene containment is necessary when producing immunogenic proteins in plants. This safety issue is particularly difficult to address in insect pollinated forage legumes, unless strictly confined cultivation is practiced, or genetic containment tools are implemented (discussed in Rosellini 2004).

5 Conclusions

Rapid developments in the area of molecular genetics and genomics have allowed to broaden our knowledge of the genetic composition of many forage and turf species

and to gain insight in the genetic control of various agronomic traits. However, despite the increasing number of studies on marker-assisted germplasm characterisation and QTL mapping, only few examples are available where molecular genetic tools have been successfully implemented in forage crop breeding programs. This may on the one hand be due to the predominantly population-based selection schemes which complicate the direct implementation of marker-assisted selection. A more extensive employment of cultivars based on genetically modified plants on the other hand has so far certainly been prevented by the limited public acceptance of the technology involved. However, genetic engineering technologies have been indispensable for functional analysis of genes. A better knowledge of gene function will support the development of markers applicable in fodder crop improvement. The tremendous recent advances in DNA sequencing technologies will lead to an easy access to whole genome sequences for many forage species and genotypes at reasonable cost in the near future. This, together with the necessary advances in data handling and statistics for trait dissection will allow for a more efficient use of genetic and genomic information in plant breeding programs.

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Breeding Objectives in Forages

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1 Introduction

All breeding programs share one common objective – to improve a species for use within a target population of environments and a particular agricultural context. Beyond this common goal, the objectives of forage breeding programs are as varied as the species upon which they are based and the breeders who develop and implement them. Many breeding objectives are determined a priori by the choice of a species with one or more obvious trait limitations or deficiencies, such as poor seedling vigor, synthesis of toxic alkaloids, or severe susceptibility to a major pest. For species without such limitations, breeders have the luxury of defining less stringent and/or more flexible breeding objectives. This may allow breeders to focus their efforts on improving breeding methods and efficiency, develop additional breeding objectives or selection criteria, or simply to increase the size or scope of the breeding program.

Breeding objectives are framed within the agricultural context and the environments in which the species will be used (Figure 1). Management and/or environmental constraints that impose stress upon a species should be used to develop breeding objectives and methodologies that allow selection for tolerance or resistance to the stresses imposed. In some cases, this can be accomplished by dissecting complex traits into simpler traits, such as using a simple freezing tolerance test to select plants with improved freezing tolerance, improved persistence in the field, and increased long-term yield stability. In other cases, certain managements or environments may obviate the need to focus on particular breeding objectives that are otherwise important. For example, toxic alkaloids of reed canarygrass (*Phalaris arundinacea* L.) are metabolized to non-toxic compounds in dry hay, eliminating the need to breed for reduced alkaloid concentrations in reed canarygrass cultivars to be used for hay production.

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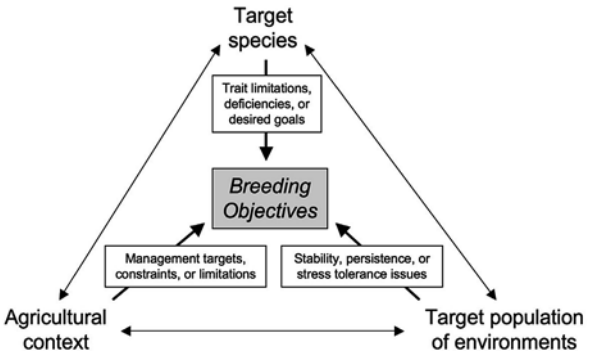

Fig. 1 Illustration of the trilateral relationship of target species, agricultural context, and target population of environments in determining forage breeding objectives

2 Growth Characteristics

2.1 Evaluation of Phenotype: Spaced Plants Versus Swards

The vast majority of forage breeding is conducted in spaced-plant nurseries, which are comprised of hundreds or thousands of plants on a regular spacing, designed for efficient collection of individual plant samples and/or data. Nearly all spaced-plant nurseries use spacings sufficiently wide to allow easy movement among plants for sample and data collection and to maintain the integrity of each genotype, eliminating competition between neighboring plants and creating a uniform environment for maximum phenotypic expression of each plant. As a result, spaced-plant nurseries bear no resemblance to the real world of forage production in swards, particularly mixed swards of multiple species, in which there are high rates of seedling and adult-plant mortality, interplant competition, and potential seedling recruitment, each of which can cause dynamic changes to sward density and composition over time.

The forage breeder must always be mindful whether a particular trait has a high, moderate, or low genetic correlation between spaced plants and swards. Some complex traits such as biomass yield per unit land area usually cannot be directly translated from spaced plants to swards (Casler et al. 1996, Wilkins and Humphreys 2003). There are some notable exceptions in which selection for increased forage yield of spaced plants led to increased sward-plot yields, but there are also numerous failures to improve sward-plot yield by this approach. In an effort to compromise and employ some level of competition, many breeders utilize very narrow plant spacings, which work well for non-rhizomatous species or spaced-plants-in-swards, in which a contrasting species is overseeded to provide ground cover and a uniformly competitive environment.

The ultimate validation is to evaluate progress from selection from spaced-plant nurseries in realistic sward plots using the original population as a control. Using this process, the use of spaced-plant nurseries has been validated for many

traits. Of these, some of the most reliable traits include simple morphological or physiological traits (e.g., flowering time, leaf size and shape, stem diameter, and plant height), forage quality traits, pest resistances, and some stress tolerances (see reviews by Abberton and Marshall 2005, Casler et al. 1996, Casler and Pederson 1996, Humphreys 2005, Wilkins and Humphreys 2003).

2.2 Seed and Seedling Traits

Many forage crops, particularly warm-season grasses, are difficult to establish, in some cases caused by inherently low seedling vigor and competitive ability and in other cases by environmental issues such as heat and drought during germination and establishment. Few forage crops have been domesticated in the sense that cultivars are easily differentiated from wild populations or that cultivars cannot survive without human inputs. As such, many forage crops still possess traits of wild plants that include seed shattering, small seed size, seed dormancy, and relatively slow germination rates, although some exhibit weedy traits such as rapid germination under most circumstances (e.g., *Lolium multiflorum* Lam.).

Seed size is moderately to highly heritable and readily amenable to selection. Seedling vigor is highly correlated between laboratory and field tests allowing the use of simplified laboratory or glasshouse selection protocols with large population sizes and uniform germination, emergence, and growing conditions. Larger or heavier seeds generally have increased seedling vigor, more rapid germination, larger seedling shoot mass, and more rapid adventitious root formation. Generally, the effect of larger seeds lasts for only a few weeks after germination with minimal effects on long-term growth of adult plants. Selection for rapid germination can also be used to reduce the proportion of dormant seeds in forage populations. Selection for reduced seed dormancy can be complicated by multiple mechanisms of dormancy including seed morphology, seed mass, thickness of lemma and palea, and genetic variation that is independent of seed morphology (Jones and Nielson 1999).

In addition to direct selection for seedling traits, selection on seedlings is an essential component for development of efficient DNA marker selection protocols. DNA markers can be rapidly and meaningfully screened on thousands of seedlings, based on marker-trait associations from phenotypic and genotypic evaluations of parental plants. Seedling screens of DNA markers, using adult-plant marker-trait associations provide a mechanism to apply selection pressure for complex traits such as biomass yield both among and within family pedigrees.

2.3 Reproductive Development

Forage plants undergo fundamental phase changes as they develop. Individual shoots proceed from the embryonic phase to three postembryonic phases: postembryonic development, juvenile, and adult (Poethig 2003). The transition from one

phase to the next is controlled by an array of independent signal transduction pathways that respond to external stimuli such as thermal time (growing degree days) and photoperiod. During their juvenile phase, forage plants are generally highly susceptible to exogenous stresses such as drought, cold, and heat, largely because they do not yet possess the hardening mechanisms required to attain their genetic potential for stress tolerance. In grasses, there is a gradual increase in leaf blade length, leaf blade width, and leaf sheath length during the juvenile phase. Timing of the juvenile–adult transition is under genetic control (Basso et al. 2008).

The transition from vegetative to flowering phase, which generally occurs only in adult plants, is regulated by genes that belong to one of four parallel pathways – gibberellin, autonomous, vernalization, and light dependent – plus an integration pathway (Cockram et al. 2007). Many of the genes involved in flowering pathways play no role in developmental regulation during vegetative phase changes. Indeed, alterations to flowering-time genes may drastically alter flowering dates, but have no effect on timing of vegetative phase changes.

Flowering time in forage crops is a highly heritable trait, controlled by genes with additive effects and easily amenable to phenotypic selection. Genes that regulate the photoperiodic control of flowering time are highly conserved across diverse species. Identification of numerous single-nucleotide polymorphism (SNP) DNA markers within genes that control flowering creates opportunities to efficiently employ marker-selection methodologies for selection on heading date well before the trait is phenotypically expressed.

2.4 Persistence

Persistence of perennial forage crops should not be considered as a single trait, but rather as a complex of traits that are each dependent on the environment and agricultural context of the crop. The greatest achievements for improved persistence of forage crops have arisen from clear definitions of the problem and a clear path toward the solution. When perennial forages demonstrate a significant lack of persistence, experiments should be undertaken to determine the cause(s) of plant mortality. Are plants dying due to disease, insects, abiotic stresses, or stress that arises from some new management regime? Once the principal source of plant mortality is identified, a selection protocol or phenotypic screen should be designed to allow plants to be uniformly exposed to the stress(es). Can this be done in the laboratory or glasshouse to improve uniformity and repeatability, or must it be done in the field for realistic assessment? Can the phenotypic screen be applied to seedlings with a reasonable expectation of selecting plants with improved adult-plant persistence?

Bacterial, fungal, and viral pathogens, nematodes, and insect pests are frequent causes of mortality in forage plants, particularly those that affect the vascular system and/or roots of the host plant. There are numerous examples of improved persistence directly resulting from selection and breeding for pest resistance (see reviews by Abberton and Marshall 2005, Casler and Pederson 1996). In most of these examples,

selection protocols have been based on uniform screens of thousands of seedlings or juvenile plants, often evaluated under artificial inoculations in the laboratory or glasshouse.

Abiotic stresses also play an important role in limiting the persistence of some forage species. Mortality can be caused by abiotic stresses such as heat, cold, drought, flooding, acid soils, salinity, heavy metals, air pollutants, and severe management regimes. Morphological traits, such as stolon, rhizome, or root characteristics, can be manipulated to reduce persistence problems caused by some abiotic agents such as heat and drought (Abberton and Marshall 2005, Casler et al. 1996). Many populations of forage plants may contain very low frequencies of genes for tolerance to one or more abiotic stress factors, challenging the breeder to design a selection protocol that will capture the few plants that possess these genes and to then concentrate them in resistant or tolerant populations.

Many perennial grasses and some legumes are host to endophytic fungi that form mutualistic relationships with their host. The fungi obtain water, nutrients, and a long-term survival mechanism from the host, at the same time producing alkaloids and other compounds that protect the host from herbivory and some abiotic stresses. Fungal endophytes (*Neotyphodium* spp.) associated with tall fescue (*Festuca arundinacea* Schreber) and ryegrasses (*Lolium* spp.) produce ergot alkaloids that are responsible for severe health problems in bovines resulting in huge economic losses for cattle producers. Because endophytic fungi can protect host plants from several stress factors, the presence or absence of the endophyte may drastically alter the breeder's objectives in some environments and managements (Pedersen and Sleper 1988). Isofrequent endophyte-containing and endophyte-free populations can almost be considered as different species as far as breeding objectives are concerned. Newer approaches to dealing with the endophyte problem in fescues and ryegrasses include deliberate infection with strains with drastically lower ergot alkaloid production and even strains where key enzymes in the ergot pathway have been knocked out.

3 Biomass Yield and Its Components

3.1 Measurement of Biomass Yield

Biomass yield is most appropriately measured on sward plots, established either as drilled rows or from broadcast seed. Where seed is limiting, closely spaced plants can be used to simulate sward plots provided that the spacing is sufficiently narrow to allow interplant competition and sufficient time is allowed for plants to spread into each other, filling the open spaces. Caution should always be used for inferences from sward plots established from closely spaced plants, because these plants represent random plants of the population or family being evaluated. Mortality rates are high in sward plots established from seed – up to 90% mortality during the establishment year – and strong selection pressure for fitness traits has been observed

within the first 2–3 years after stand establishment. Due to mortality and selection, the genetic and phenotypic composition of both grass and legume sward plots can change rapidly so that sward-plot evaluations of families or breeding lines are generally not evaluations of random plants from a bag of seed. Other methods of assessing biomass yields include row plots or spaced plants, using a fairly wide spacing between rows or plants, but the potential pitfalls of widely spaced plants were discussed earlier.

The size of sward plots varies considerably among forage breeding programs, usually determined by the seeding and harvesting equipment available. Because seed of families or breeding lines is generally limiting, plot sizes for family selection protocols are generally smaller than for cultivar evaluations. With an appropriate experimental design and statistical analysis, small sward plots (1.2 m^2) can be very effective for half-sib family evaluations of breeding value.

Experimental designs for sward-plot evaluations of families can range from simple randomized complete blocks to a wide array of incomplete block designs. The latter group includes blocks-in-reps (a variation of the split-plot), many types of lattice designs, and α-designs. Because blocking designs rely on the breeder's ability to predict patterns of field variation and lay out blocks accordingly, postdictive spatial analyses are often useful supplements to experimental design for controlling unexplained spatial variation and providing accurate estimates of family means. Trend analyses or nearest neighbor analyses have been simplified within mixed models frameworks so that they can be easily incorporated into the analysis of many families evaluated over multiple years and locations (Smith and Casler 2004).

3.2 The Role of Environment and Management in Measuring Biomass Yield

Biomass yield is one of the most important traits of forage plants, acting as the single unifying trait that is measured in nearly every cultivar evaluation trial, regardless of the environment or agricultural context. The most appropriate measurement of biomass yield depends on the agricultural context of the forage crop. Management schemes may range from one conservation harvest per year for warm-season bioenergy grasses to continuous stocking of grass–legume pastures, including nearly any intermediate timing and frequency of cutting or grazing. For conservation managements, a machine harvester is generally used to harvest forage plots, cutting the crop with a sickle-bar mower or a flail chopper. Harvest managements and timing will vary among species and location, but should always be designed to accurately reflect real-world practices in the target region for the forage crop. The old breeder's axiom should always be remembered, "What you select is what you get."

Conversion of fresh-matter forage yields to dry-matter forage yields is problematic for large replicated trials containing hundreds of families and multiple locations, cuttings, and years. For forage species or breeding populations of the same ploidy

level and in which maturity is relatively constant, fresh-matter and dry-matter forage yields are highly correlated so that fresh-matter forage yield can be used as a surrogate for dry-matter forage yield. This decision must be made with great care and thought, because genetic variation for growth stage or flowering time could confound the breeder's ability to make genetic progress when dry-matter concentration is negatively correlated with fresh-matter forage yield.

The methodology for measurement of forage yield under grazing management is much more variable than under conservation management. Grazing managements are often simulated by machine-harvesting sward plots on a schedule that simulates local grazing managements. However, simulated grazing management by frequent machine harvesting captures only the first of four major effects that grazing livestock may exert on pasture plants: (1) frequent defoliation and the requirement for frequent regrowth and regeneration, (2) selectivity of plants that may favor persistence of specific genotypes, (3) trampling that may damage plant tissue and compact the soil, and (4) excretion that concentrates nutrients in small areas affecting palatability and nutrient cycling (Hart and Hoveland 1989). The last three of these effects create the need for larger plot sizes and more replicates than typically used for large-scale family evaluations under conservation management, largely because of the additional within-plot variability induced by livestock. In a rotational grazing scheme, higher stocking rates and shorter grazing times can be used to increase uniformity of grazing management within and among breeding plots.

Under grazing management, herbage mass can be measured directly (destructive sampling) or indirectly (nondestructive sampling). Direct measurement of herbage mass involves the use of a quadrat or sampling frame and hand harvesting all herbage within the frame to a specific cutting height at or below the desired grazing height. For estimates of total herbage mass, cutting height should be at the soil surface. Indirect measures of herbage mass are classified into three groups: visual, height and density, and nonvegetative attributes. For quantification of herbage mass, all indirect measures must be calibrated by the use of a double-sampling technique, preferably repeated over time and space to capture environmental variation.

Employing the previously mentioned breeder's axiom, it should be obvious that different plants will be selected under different management schemes, depending on the traits that favor persistence, recovery, stress tolerance, and/or high herbage mass. Empirical studies have demonstrated that there is often little or no correlation between biomass yield measurements under highly divergent management schemes, particularly for management schemes that place different stresses upon the plants such as cutting versus grazing, i.e., genotype × management interaction. Many breeders have attempted to develop grazing-tolerant alfalfa (*Medicago sativa* L.) by either trait selection or selection of plants under frequent mowing – all of these efforts failed to produce a grazing-tolerant alfalfa. In contrast, selection of alfalfa plants that survived several months of continuous stocking resulted in germplasm with consistently high grazing tolerance under both continuous and rotational stocking. High forage yield of the grazing-tolerant alfalfa cultivars under conservation management indicates that the addition of the grazing tolerance trait has broadened the adaptation of this germplasm (Bouton and Gates 2003).

Because most forage crops are grown in mixed swards, often including at least one grass and legume species, there has been considerable effort to define how best to select plants for competitive ability or combining ability with companion species, sometimes referred to as coexistence. Trait breeding in pure swards – flowering date, tiller density, canopy height, and leaf traits (leaf length, leaf size, petiole length) – may be effective for developing either grass or legume germplasm compatible with companion species in mixed swards. Alternatively, strong interactions between genotypes and sward types (pure versus mixed swards) in many studies, suggesting complex mechanisms that regulate competition for space, validate the breeder's axiom that selection for performance in mixed swards should be conducted in mixed swards.

3.3 Breeding for Increased Biomass Yield

Gains made in biomass yield of forage crops vary widely among regions and species, generally ranging from about 1 to 6% per decade (Humphreys 1999, Wilkins and Humphreys 2003) but gains may be dependent on environment and management. Pyramiding multiple pest resistances is a mechanism for improving persistence of alfalfa, but decades of breeding for multiple pest resistances in alfalfa resulted in no change to biomass yield potential in the absence of significant disease pressure (Lamb et al. 2006). Similarly, significant gains made in biomass yield under hay management may not be observed under management-intensive rotational grazing, due to the absence of livestock during the selection process.

Gains in biomass yield of forage crops have largely resulted from selection of superior families and recombination of parental clones or selected plants of the best families. (Details of family-based breeding schemes are provided in Chapter 3.) Intensive selection for increased biomass yield of sward plots can lead to rates of gain as high as 10% per decade (Wilkins and Humphreys 2003). However, family selection based on sward plots carries a significant cost in the relatively large number of families that must be continually carried through the breeding program and the large number of plots that must be repeatedly harvested. For those species in which spaced-plant and sward-plot biomass yields have a positive genetic correlation, phenotypic selection of spaced plants can be used to effectively increase biomass yield of sward plots. Generally, gains measured in sward plots are generally reduced to half or less than those observed on spaced plants, because selection was conducted under non-competitive conditions and the genetic correlation between competitive and non-competitive conditions is less than one.

Indirect selection has often been employed to improve biomass yield per se, but none of these efforts have led to long-term sustained gains in biomass yield. Some of the traits that have shown promise as indirect selection criteria for biomass yield of forage grasses include specific leaf weight, leaf length, leaf area expansion rate, mesophyll cell size, and dark respiration rate (Casler et al. 1996). Indirect selection for stolon and leaf traits of white clover has probably had some positive

impact on biomass yields of white clover (Abberton and Marshall 2005, Woodfield and Caradus 1994). The general conclusion is that if one aims to improve biomass yield one should select based on biomass yield, which is only restating the breeder's axiom mentioned above in a slightly different form.

3.4 Seasonal Distribution of Biomass Yield

Improved seasonal distribution of biomass yield has long been a goal of forage breeders and agronomists. Extension of the growing season, either by early-spring growth or late fall growth, or more uniform production throughout the growing season has been the most common target. Non-uniform dry-matter production throughout the growing season is typically tied to reproductive growth – plant biomass increases up to a certain point associated with flowering, when seed formation, remobilization of storage carbohydrates, and leaf senescence lead to reductions in plant biomass. For cool-season forages, particularly grasses, this results in a "summer slump" in which biomass production is significantly reduced during the warmest period of summer, often to the point of dormancy for some species.

Early flowering types of cool-season grasses and legumes have a more even distribution of dry matter throughout the growing season creating a longer post-flowering growth period in humid, temperate regions. Non-flowering or sparse-flowering populations have been developed in some forage grasses with the goal of increasing nutritional value, simplifying grazing management, and creating a more favorable seasonal distribution of forage yield compared to normal-flowering populations. In timothy (*Phleum pratense* L.), early-heading genotypes with a high frequency of flowering tillers on regrowth harvests had the highest biomass yields in summer and for the entire growing season, resulting in a more uniform distribution of biomass production.

4 Nitrogen Economy

4.1 Nitrogen Fixation of Legumes

Forage legumes fix atmospheric nitrogen (N_2) as a source of internal nitrogen nutrition and as sources of nitrogen nutrition for companion grasses or a subsequent crop in a crop rotation system. N_2 fixation can be measured directly by the acetylene-reduction assay, the difference method (total reduced N of a treatment versus a non-N_2-fixing control), or the ^{15}N isotope dilution method. The acetylene-reduction method has been used to develop alfalfa genotypes and cultivars with significantly improved levels of N_2 fixation, repeatable in multiple field environments. This research has led to the development of alfalfa cultivars with high N_2 fixation and biomass yield for use specifically as an annual plow-down crop in crop

rotations. Indirectly, N_2 fixation can be improved by selection for high tissue N concentration in inoculated legume plants grown on a N-deficient soil. Recent research with ^{15}N isotope dilution indicates that the application of N-fertilizer to mixed white clover (*Trifolium repens* L.) – perennial ryegrass (*Lolium perenne* L.) stands increases N-transfer from the legume to the grass. The mechanism is greater rooting in the grass component and hence, better access to the N deposited by legume roots; N-fixation but not exudation (ammonium and amino acids) is depressed by N-application (Paynel et. al, 2008).

4.2 Nitrogen Use Efficiency of Grasses

Nitrogen use efficiency (NUE) can be improved in grasses by selection for increased biomass yield under uniform soil-N conditions. The levels of soil-N and applied fertilizer should be closely aligned with the intended agricultural context of the crop. If the crop is to be grown under conditions of relatively low N nutrition, as is often the case with perennial grasses, selection should be conducted under these conditions to identify plants capable of scavenging N from these soils. Under these conditions, increased biomass yield will arise from a reduction in tissue N concentration and/or an increase in the proportion of available soil N recovered by the plants (Wilkins and Humphreys 2003). Tissue N concentration is moderately to highly heritable in perennial grasses, so selection for combined high biomass yield and high tissue N concentration may be an effective method of indirect selection for more efficient N uptake as a mechanism to improve NUE.

5 Forage Quality

5.1 Laboratory Estimators of Forage Quality

Breeding forages with improved quality requires laboratory assays that are rapid, repeatable, heritable, and are directly correlated with animal performance. *In vitro* dry-matter digestibility (IVDMD), including various modifications of the original Tilley and Terry (1963) procedure, was the first of these assays to receive overwhelming support from the scientific community. Breeding efforts for increased IVDMD were initiated almost immediately after publication of the Tilley and Terry procedure. The in situ nylon bag dry-matter digestibility (NBDMD) procedure was one of the earliest and most highly successful modifications of the original assay. The attractiveness of the IVDMD procedure derived largely from its direct reliance on rumen microorganisms for tissue degradation and its role in ruminant nutrition, directly through *in vivo* digestion and indirectly through a positive impact on voluntary intake.

Traits such as IVDMD are determined by fundamental chemical and physical traits of the plant, including cell-wall structure and composition, anatomical

composition of organs, and organ morphology. Four mechanisms to increase IVDMD of forage plants have been demonstrated: (1) decreased fiber or cell-wall concentration, (2) increased concentration of water-soluble carbohydrates (WSC), (3) decreased lignification of the cell wall, and (4) decreased ferulate cross-linking between lignin and polysaccharides in the cell wall (Casler and Vogel 1999, Casler 2001). The first two of these mechanisms probably overlap with each other – without an increase in net photosynthesis there is a fixed carbon pool that is allocated between the soluble and structural carbohydrate pools, theoretically resulting in a negative correlation between structural and soluble carbohydrate concentrations. The latter two mechanisms operate independently of each other, both acting to increase IVDMD and, more importantly, digestibility of the fiber or cell-wall fraction per se.

Laboratory measures of intake potential have been much more problematic for forage agronomists and breeders, largely because voluntary intake measurements are more susceptible to inherent variability and are less directly related to measurable plant traits. The concentration of neutral detergent fiber (NDF), a simple and inexpensive assay, has long been considered as the single laboratory variable most directly related to voluntary intake of ruminants (Casler and Vogel 1999, Casler 2001). Other measures of intake include physical measurements such as particle-size breakdown by ball milling or artificial mastication, the energy required to grind a forage sample to pass a given mesh size, and the energy required to shear an intact leaf blade, respectively (see reviews by Casler and Vogel (1999) and Casler (2001).

5.2 Breeding Methods and Breeding Progress

Most laboratory traits that predict forage quality are highly stable and repeatable across a wide range of environmental conditions. They tend to be far less sensitive to genotype × environment interactions than agronomic traits such as forage yield and stress tolerances. Most breeding activities begin with a spaced plant nursery, allowing easy harvest, identification, and eventual recovery of individual plants. Samples are collected from each plant, usually by hand harvest, in a uniform manner and processed as uniformly as possible throughout the stages of drying, grinding, and analysis. Traditional methods of analysis would involve direct assay of each sample by wet laboratory methods, but that process has been largely streamlined by the use of near-infrared reflectance spectroscopy (NIRS). In most breeding programs, samples are scanned using NIRS and a broadly representative subset of samples is analyzed, using wet chemistry methods. Prediction equations are developed from the calibration set and used to generate predicted values for the entire breeding nursery.

The use of NIRS in a breeding program is highly cost-effective, reducing the need for wet chemistry to a very small proportion of the total samples, sometimes as low as 10%. Calibration equations can be developed using either open or closed population calibration methods. Open population calibration involves development

of a broad calibration set, typically for one species or group of similar species, with occasional new samples added to the calibration set to represent new environments, genotypes, or growing conditions. Closed population calibration involves development of calibration sets for specific experiments or selection nurseries, with no attempts to develop broader calibrations across space or time. As NIRS technologies, statistical methods, and software have improved, open population calibration methods have become more accurate and precise and are gradually replacing closed population methods in many breeding programs.

Once the most favorable plants are identified, they are typically removed from the original nursery and transplanted to crossing blocks in the field or in isolated crossing houses. Crosses can be made between individual plants with superior levels of a trait or among a larger group of plants, depending on the breeding methods being employed. In a recurrent selection program, the resulting seed is used to establish a new nursery to begin the next generation or cycle of selection.

Two critical factors have simplified many breeding schemes designed to improve quality of forage crops. First, heritability of many forage quality traits (e.g., IVDMD, NDF, WSC, crude protein, plus many others) is moderate to high for selection on the basis of an individual unreplicated plant. This principle has been verified many times, most effectively by completion of one or more cycles of selection for a trait, followed by evaluation in a new series of experiments, typically under swardplot conditions. The moderate to high heritability of most forage quality traits has been verified by positive and significant genetic gain, as indicated by superiority of a new population compared to the original population from which it was derived, in a new experiment under different growing conditions from the original nursery (typically sward plots versus spaced plants). Second, genetic gains made for most forage quality are stable under a wide range of conditions, including different growing conditions, growth stages, harvest managements, and soil types. Realized gains for WSC and IVDMD of perennial ryegrass have demonstrated stability from spaced plant nurseries to sward plots to on-farm trials under grazing livestock (Humphreys 1989, Walters 1984).

Genetic gains in IVDMD, WSC, and other traits related to digestibility and intake potential of forage crops may accrue very rapidly, with published results indicating gains of 5–66 g kg^{-1} $cycle^{-1}$ (Casler 2001). Most selection programs are capable of completing a cycle of selection within 2 or 3 years, depending on the vernalization requirements of the species, the need to assess stress tolerances or fitness of the selected plants, and the amount of seed required for the next generation of selection. The use of significant modifications to the selection methods and personnel dedicated to this effort at critical times may allow a reduction in generation time to 1 year for species that can be forced to flower within that time and for which there are no stress tolerance or fitness issues.

Despite the ready availability of efficient selection criteria that are related to animal performance and have excellent laboratory repeatability, relatively few breeding programs are dedicated to improving forage quality. For forage crops with relatively high inherent quality other traits and objectives demand the most attention. In other cases, there are still many perceptions that breeding for forage quality is a sure way

to decrease forage yield and other fitness traits of forage crops. Thorough review of the literature indicates that fitness problems associated with genetic improvements in forage quality are very much the minority (Casler 2001). There have been reports of reductions in lignin concentration associated with decreased cold tolerance and increased disease susceptibility, but other reports have shown that these problems can generally be overcome by concomitant selection for fitness traits in the field to ensure that these traits are not lost during the selection process. Among forage quality traits that are controlled by multiple genes with relatively small individual effects, what we normally describe as quantitative genetic variation and quantitative trait loci (QTL), only NDF has shown consistent fitness problems across multiple populations and species. Reduced NDF has been consistently associated with reduced forage yield and detailed genetic analyses have indicated that this is due to multiple causes that include genes with multiple specificities, linkage between genes controlling both traits, and random processes such as drift (Casler, 2005).

5.3 Anti-nutritional Factors

Many types of plant compounds and structures can be detrimental to utilization of forage crops by livestock, largely by reducing palatability, digestibility, intake, and/or health and fitness of livestock. These traits exist largely as defense mechanisms in plants that have coexisted with mammalian herbivores for eons, all designed to reduce herbivory, while herbivores evolved traits to overcome these defense mechanisms, including mouth and tooth structure, rumination, multi-chambered fermentation systems, and diverse microbial populations.

Most defense mechanisms are chemical in nature, although there are examples of physical or structural defense mechanisms, such as trichomes or siliceous dentations on leaves or stems. Chemical defense mechanisms include toxins, estrogenic compounds, and narcotic compounds, such as alkaloids. Alkaloids and cyanogenic compounds that contain nitrogen may serve the dual roles of defense against herbivory and sequestration of nitrogen. Saponins, tannins, estrogens, and cyanogenic compounds are common in many important forage legumes. While tannins are known to cause reductions in palatability of some legumes, they are also thought to play an important role in binding soluble proteins in the rumen, helping to protect ruminants against bloat. Genetic variability and relatively simple inheritance patterns have been demonstrated for many of these compounds, which are fairly amenable to selection. Selection for low levels of the estrogen formononetin in red clover (*Trifolium pratense* L.) resulted in significant improvements in fertility and breeding times of ewes (*Ovis aries*) (McDonald et al. 1994). Condensed tannins in sericea lespedeza [*Lespedeza cuneata* (Dum. Cours.) G. Don] and sainfoin (*Onobrychis sativa* L.) confer two advantages to small ruminants: control of internal parasites such as *Haemonchus contortus* and increased bypass protein, both resulting in increased animal performance.

Natural populations of reed canarygrass and phalaris (*P. aquatica* L.) contain a wide array of alkaloids that can have toxic and narcotic effects on livestock, reducing palatability, intake, digestibility, health, and fitness. Two genes control the type of alkaloid, simplifying the process of identifying and intercrossing true-breeding plants without the highly toxic compounds in the tryptamine and β-carboline chemical families. The less-toxic alkaloid, gramine, causes little more than reductions in palatability, which have been solved by screening large populations of individuals for gramine concentration in the process of producing low-gramine cultivars. Grazing trials have demonstrated that elimination of tryptamines and β-carbolines reduces animal disease issues, while reductions in gramine concentration increase palatability, intake, and liveweight gain (Marten 1989).

Mineral imbalances, such as inadequate or excessive levels of one or more mineral elements, can be classified as anti-nutritional factors without necessarily being anti-herbivory defense mechanisms. Hypomagnesemia is the most serious disease caused by a mineral imbalance, specifically a deficiency of Mg or an excess of K in fresh herbage. Because mineral elements such as Mg and K are under genetic control, selection for increased Mg and/or reduced K concentrations have led to the development of new cultivars with reduced potential for this disease. Grazing studies have demonstrated significant gains in blood serum Mg and reductions in livestock fatalities in direct comparisons of high-Mg versus low-Mg cultivars (Moseley and Baker 1991).

5.4 Livestock Evaluations

A demonstration of increased animal performance is the definitive proof of impact from breeding forage crops for increased forage quality. As mentioned above, this has been accomplished in several instances of selection against anti-quality factors. Yet, although there are few reports of successful validation from grazing or feeding trials, there is convincing evidence that genetic increases in IVDMD, WSC, or related traits will generally positively impact livestock performance (Casler and Vogel 1999).

Increased IVDMD has a direct and positive impact on average daily weight gains of livestock. This effect is typically greatest for warm-season grasses which generally have the lowest forage quality, providing a greater potential benefit for the same investment in selection and breeding. Because most new cultivars with genetic increases in forage quality also represent increases or no change in forage yield, relative to their parent cultivars, increased liveweight gains per animal typically translate to increased liveweight gains per hectare. Despite their expense, grazing and/or feeding trials have a proven benefit in supporting efforts to market and distribute new cultivars. The three most successful forage breeding programs dedicated to improving forage quality for at least 25 years – the USDA-ARS bermudagrass [*Cynodon dactylon* (L.) Pers.] program at Tifton, GA, USA; the USDA-ARS warm-season grass program at Lincoln, NE, USA; and the UK ryegrass program at Aberystwyth, Wales – all rely on grazing experiments to document the effect on livestock

performance. Often, these animal performance data are largely or solely responsible for the success of new cultivars.

6 Biotic and Abiotic Stresses

6.1 Breeding for Durable Pest Resistance

The perennial nature of many forage crops, the use of many species in monocultures, and the broad range of environmental conditions over which many forage species are grown all contribute to pest problems. Many of these pests reduce forage yield and/or quality, may contribute to reduced livestock performance or health, and result in reduced vigor/significant mortality of forage plants. Breeding for pest resistance is perhaps one of the most common objectives in forage breeding. It could be argued that all forage breeders participate in this activity, at a minimum by culling highly diseased plants within selection nurseries, even when disease resistance is not the primary breeding objective. In alfalfa and red clover, genetic gains for multiple pest resistances represent the most significant historical breeding progress, leading to improvements in persistence and stand longevity, forage yield, and economic returns (Elgin 1985, Smith and Kretschmer 1989).

Phenotypic recurrent selection is by far the most common selection method used to improve pest resistance in forage crops. This selection process involves development of a uniform screening process, ensuring that all plants are grown in a uniform environment and that pathogen inoculum is uniformly delivered to each plant. Many of the largest forage breeding programs will routinely screen over 1 million plants per year for disease resistances.

For these programs, and for many smaller programs, the use of indirect selection methods is an absolute requirement. Indirect selection involves selection for a trait that is not exactly the target trait, but is positively correlated with the target trait to such a degree that the losses associated with selection for a different trait are more than offset by the gains associated with improved selection efficiency and intensity. For example, phenotypic selection for increased yield will invariably lead to the elimination of susceptible genotypes for temperate forages grown in a subtropical humid environment. The use of seedlings that may still be in a juvenile developmental phase and glasshouse screening environments create highly uniform, but artificial, conditions for pathogen development. Nevertheless, uniformity of both the environmental conditions and the delivery of pathogen inoculum, combined with the potential to screen many more genotypes than could be screened in the field, has resulted in realized genetic gains for many pathogens of many species (Casler and Pederson 1996). While there are many success stories, there are some examples in which seedling screens in the glasshouse were either not successful or less efficient than field selection, due to low correlations between the glasshouse and the field and/or seedlings and adult plants. Research on these relationships should be undertaken in all circumstances to determine the most efficient selection methods

and the trade-offs associated with indirect versus direct selection. Indirect selection for forage yield or vigor in the presence of uniform pathogen loads can be a very effective method of improving resistance to viruses or nematodes in forage populations.

Phenotypic recurrent selection does not require the presence of "resistant" plants in the population targeted for improvement. Resistance genes may often be present as hidden recessives in extremely low frequencies, leading to the presence of clearly resistant plants only after two or three cycles of selection have increased the frequency of resistance alleles in the population and created plants homozygous for these alleles. Once the first resistant plants appear in a population, the rate of progress may rapidly increase due to selection of a higher frequency of resistant plants each of which carry higher doses of resistance alleles.

Many pathosystems are governed by host–pathogen specificity in which gene-for-gene relationships regulate the disease phenotype and host genotypes do not respond uniformly to all isolates or races of a pathogen. It is clear from thorough review of the literature that these gene-for-gene relationships exist in many forage pathosystems, but it is also clear from the many successful selection experiments that selection for resistance to a single pathogen isolate or a simple mixture of a small number of isolates leads to success in the vast majority of cases (Casler and Pederson 1996). The goal in most breeding programs is to utilize isolates that are present in field conditions and are highly pathogenic to the host, placing maximum selection pressure on the host population without killing all host plants.

Genetic regulation of host resistance varies from single major genes to oligo- or polygenic resistance. In theory, multiple-gene resistance should be more durable than single-gene resistance. A number of examples of long-term durability of single-gene resistance exist within forage pathosystems (Casler and Pederson 1996). Because most forage species are highly self-incompatible, largely outcrossing, and composed of highly heterogeneous populations of highly heterozygous host plants, a tremendous amount of genetic diversity is maintained within most cultivars. This is particularly true for polyploid species. Breeding for increased pest resistance increases the frequency of resistance alleles, resulting in frequencies of resistant plants of 30–60%, seldom more than 70%, in finished cultivars. These percentages are typically sufficient to withstand moderate mortality rates that occur due to pathogens and other stresses, while resistant plants are capable of compensatory growth to help fill in empty spaces created by mortality of susceptible plants. Such a system allows some level of coexistence between the host and pathogen, reducing selection pressure on particularly virulent strains of the pathogen that may arise from mutation or genetic recombination, leading to some very durable host resistances.

6.2 Moisture Stress

Drought tolerance is a very elusive trait in many species, without obvious phenotypic variation upon which to base selection. While it may seem to be the simplest and most obvious approach, simply withholding or reducing water for a certain time

period, either in a glasshouse or with field-based rainout shelters, often fails due to lack of uniformity in application of drought, low heritability, or inability to predict a drought regime to optimize selection pressure.

Many approaches to improve drought tolerance have utilized indirect selection. Some of the most promising of these traits include high stomatal resistance (less frequent stomata, smaller stomata, or shallow epidermal ridges), low leaf water conductance, low osmotic potential, and low C isotope ($^{13}C/^{12}C$) discrimination ratio (Casler et al. 1996, Johnson and Asay 1993). Selection experiments for these traits have led to populations of plants with potentially improved drought tolerance, assessed as increased water-use efficiency or soil moisture levels, but these efforts have not yet led to commercial cultivars with improved drought tolerance.

Many programs have specifically targeted germplasm collection from regions that have historically been drought prone, hoping to capitalize on hundreds or thousands of generations of natural selection for drought tolerance. North African populations of perennial ryegrass appear to offer some potential in this regard. Directed natural selection also offers some promise as a viable approach, in which selection is based on long-term survivorship of plants on drought-prone soils. A combination of methodical and natural selection methods has been used in one of the most successful selection programs for drought tolerance of grasses used in dryland agriculture of western North America. Selection for rate of emergence from deep planting (5 cm) has been successful in several species, with positive correlated responses for emergence percentages and first-year forage yields in the establishment year for multiple field sites.

Interspecific hybrids and trait introgression are a mechanism of transferring drought tolerance from one species into a more agronomically or nutritionally desirable species. Transfer of drought tolerance from Kura clover to white clover and from meadow fescue (*Festuca pratensis* Huds.) to perennial and Italian ryegrass (*L. multiflorum* Lam.) have created populations with improved drought tolerance and many of the traits of the desirable parent (Abberton and Marshall 2005, Thomas et al. 2003).

Susceptibility to flooding can be caused by two factors: the direct effect of anoxia or susceptibility to oxygen deprivation and/or the indirect effect of increased pathogen load (increased inoculum loads, unique pathogen species, or increased pathogen diversity) in chronically wet soils. Forage breeders routinely use chronically wet soils known to have high pathogen loads to utilize a combination of natural and methodical selection for resistance to these pathogens and, indirectly, increased tolerance to wet soils. Most research on flooding tolerance of forage crops has focused on species selection, with a limited amount of research demonstrating genetic variation among cultivars and genotypes for flooding tolerance.

6.3 Temperature Stress

Cold or freezing tolerance is one of the most important factors limiting the adaptation and long-term survival of many forage crops. Low-temperature stress

interacts with other stresses, such as grazing pressure, snow molds, drought, and dessicating winds, often complicating the development of efficient screening procedures. Furthermore, low-temperature stress is conditioned by hardening plants to lower temperatures, while winter or early-spring thaws can deharden plants to reduce their low-temperature stress tolerance. Snow cover also interacts with low-temperature stress tolerance by protecting plants from severe cold. Forage germplasm with superior low-temperature stress tolerance often can be found in climates with severe winter temperatures, except where snow cover is frequent and consistent.

Several mechanisms for improving low-temperature stress have been identified in forage crops. Enhanced resistance to winter diseases such as snow molds and some root or crown rot organisms have resulted in healthier plants that are better able to withstand low-temperature stress. Genetic increases in WSC may enhance low-temperature stress tolerance, partly as a direct effect of WSC on spring recovery and partly as an indirect effect of WSC on tolerance to snow mold fungi. Unsaturated fatty acids and vegetative storage proteins have also been implicated as potential mechanisms for enhancing low-temperature stress tolerance in perennial forages. Photoinhibition can directly cause low-temperature stress, occurring during periods of high light and low temperature when the rate of light harvesting by PSII exceeds the capacity for electron transport. Androgenic plants derived from *Festuca pratensis* × *L. multiflorum* hybrids have revealed multiple genetic pathways that reduce the effects of photoinhibition during low temperatures (Rapacz et al. 2004). Morphological traits, such as stolons and rhizomes, may also act as a mechanism to enhance low-temperature stress tolerance in some perennial forage species.

Selection for low-temperature stress tolerance can be carried out effectively under artificial conditions. Plants must be systematically hardened prior to the onset of stress and these tests typically require a range of temperatures to accurately assess the potential range in responses within a diverse population. Selection is often based on LT_{50}, the temperature predicted to kill 50% of the tillers on a plant. In many perennial forage species, juvenile plants are incapable of undergoing cold hardening, forcing breeders to screen adult plants. Selection for low-temperature stress tolerance of seedlings may improve seedling tolerance levels, but it will likely have little impact on adult-plant tolerance levels (Hides 1979). Because low-temperature stress screening can be highly variable, plants are often vegetatively propagated or cloned for replicated screening of genotypes in time or space.

Tolerance to high temperatures can be identified using either field- or glasshouse-based screening procedures. In the glasshouse, repeatability, uniformity, and reproducibility of the screening procedure can be enhanced by the use of heating elements embedded in sand benches. While it is not documented per se, the gradual movement of cool-season species such as tall fescue, alfalfa, and red clover to lower latitudes of the southern USA has probably resulted from extensive, long-term, field-based breeding programs in Florida, Georgia, Alabama, and Texas that have selected for increased high-temperature stress tolerance, either consciously or unconsciously.

6.4 Chemical Stresses in Soils

Significant breeding efforts have been undertaken to increase acid tolerance of alfalfa, white clover, perennial ryegrass, and phalaris. Several selection protocols have yielded positive results, including (1) field-based selection on highly acidic soils, (2) relative root growth of seedlings exposed to solution culture with relatively high Al concentration, (3) relative root growth of seedlings grown in a soil-on-agar medium (seeds planted between agar and an 8-mm layer of acidic soil), and (4) transformation of alfalfa with a bacterial citrate synthase gene. Laboratory protocols tend to be preferred, because of greater control over environmental conditions, leading to increased uniformity of selection pressure, higher repeatability, and more reliable genotype assessments. A number of candidate genes homologous to known Al-tolerance genes in other plant species have been identified in alfalfa (Narasimhamoorthy et al. 2007).

Natural genetic variation exists for salt tolerance in a wide range of forage species, so breeding for salinity tolerance is generally a relatively simple matter of developing an effective screening method combined with a few cycles of selection to increase the frequency of favorable alleles and tolerant plants. The most effective screening procedures are generally based on rapid germination or rapid root growth in a saline solution, although there are a few examples of genetic variation among germplasm collections directly associated with salinity of the local environment (Casler et al. 1996). Progeny of plants selected for germination or root growth in saline solution culture generally breed true for salinity tolerance, and demonstrate improved performance, including germination, vigor, and forage yield under saline field conditions (Jensen et al. 2005). Even though genes for salinity tolerance may be present in extremely low initial frequencies within breeding populations, intensive selection pressures with large population sizes can lead to tolerant populations.

Observed tolerances to heavy metals have largely arisen by natural selection of a very small number of plants capable of surviving on toxic soils associated with mine spoils, smelters, and electricity pylons (Casler et al. 1996). Investigation of neighboring populations has routinely demonstrated frequencies of alleles for heavy metal tolerances as low as 0.0001 in some populations, sufficiently high for a very small number of plants to survive. Most heavy metal tolerances are simply inherited by a single locus so that tolerant plants typically breed true and can be used to create tolerant populations, many of which have been used to renovate contaminated soils. Because several heavy metals share similar uptake mechanisms, many genes for heavy metal tolerance have specificities for multiple elements, increasing the potential value and scope of heavy metal-tolerant populations.

7 Interrelationships Among Breeding Objectives

The ultimate choice of breeding objectives depends on many factors. Breeders must often make difficult decisions with little scientific information of direct relevance

to the specific objective. Practical plant breeders are much more than people who develop new cultivars – they are problem solvers. The use of forage breeding to solve forage production problems requires sufficient scientific knowledge to identify the problem, prediction of a potential solution with a reasonably high degree of certainty, identification of reasonable and reliable breeding methods and traits, and the presence of sufficient genetic variability to create new germplasm that is sufficiently improved to assist in solving the problem.

Potential breeding objectives should be weighed against alternative solutions to production problems. Are forage producers trying to grow a particular species in an environment to which it is not adapted? If so, is it worth the breeder's time, effort, and funds to solve this problem with selection and breeding? Because genetic variability for many physiological plant traits is often hidden to us until we design the appropriate screening procedure, we often cannot predict the probability of success of new breeding objectives and ventures. In many cases, there are more cost-effective agronomic, production, or management solutions to the production problems associated with a particular species, one of the simplest being to choose different species.

Setting priorities among potential breeding objectives requires the breeder to conduct an assessment of gain versus risk, even if this is conducted informally or with little scientific input. The breeder's personal knowledge and skills, availability and talents of support personnel within the breeding program, the physical facilities and equipment available to the breeding program, and the long-term funding prospects all factor into an effective assessment of gain versus risk. Many of the plant traits discussed above have received considerable attention largely because they are moderately to highly heritable, they are simple and easy to assess, they are relatively inexpensive to measure (allowing application in multiple species and/or breeding populations), and their potential impact on forage production can be assessed with relatively simple and effective methods. Many disease resistances and some stress tolerances fit into this category. On the other hand, some complex traits such as "persistence" have required many years of effort and inputs from many scientists to develop effective screening procedures, effectively reducing the risks associated with these particular objectives in a breeding program, e.g., freezing tolerance and grazing tolerance as discussed above.

Compounding these factors, many breeding objectives are interrelated to each other in complex ways, often governed by genetic correlations that result from close linkages between genes or genes with multiple specificities. Breeding objectives that are too narrowly focused may often result in unintended consequences, such as reduced fitness associated with selection for increased forage quality (Casler 2001) or reduced root growth associated with long-term selection for increased forage yield (Gates et al. 1999). Obviously, forage breeders cannot afford to measure all traits that are potentially important in the production system of a forage crop, but selection systems should be designed to minimize the effects of random genetic drift, relaxation of selection pressure for fitness-related traits, and known genetic correlations with potentially negative production implications. Supplementation of screening methods based on artificial or controlled environments with field

evaluations of selected individuals is an effective mechanism to minimize the risks associated with these pitfalls.

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Breeding Objectives in Amenity Grasses

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1 Introduction

The vast range of natural adaptation and wide distribution of the grass family, together with an inherent affinity with open landscapes has naturally led to a capability and desire to improve this natural resource to be incorporated into, and optimised in, the lifestyles of urban-dwelling man.

Grass plants are ideally suited to withstand continuous trampling and damage with their apical and axillary meristems occurring close to ground level. Their close co-evolution with grazing animals (Stebbins 1981) has made them highly adapted to close grazing (or cutting) and recuperative growth capacity after damage and biomass removal through impact with herbivore, or, indeed, sports player/turfgrass manager even if a large proportion of grass biomass is removed.

References to lawns in pleasure gardens are found in early written literature including biblical writings. These lawns were thought to be made up of low-growing flowering plants in the Persian and later Arabian gardens. Subsequently the Greeks and Romans adapted the Persian lawn gardens to their culture.

The development of grass as a lawn is a relatively recent phenomenon emerging in the 13th century, not only as an ornamental feature but as a surface for bowling and a precursor to cricket. More sophisticated gardens and bowling greens were developed in the 16th century. Golf flourished initially on natural upland and coastal turf composed largely of *Festuca* and *Agrostis* species, around 1500, the 'mowing' being provided by sheep.

Many towns and villages in Europe had a green, common or heath that served as a park and recreational area, often hosting travelling fairs, markets and games. A form of football was played on these public greens. In approximately 1660 Francis Willughby published a 'Book of Games' (Cram et al. 2003) in which he described the game of football in close to its current form.

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The great houses built in the 17th and 18th centuries were designed with large lawns, and 'grass walks' were highly fashionable. It was recommended that they were cut as often as there was the least hold for the scythe and rolled regularly. Lawns were the preserve of the very rich as they were an extravagant use of land and were labour intensive. In 1830 Edwin Budding of Gloucester developed the first mowing machine (based on a machine used to trim cloth), which went into production in 1832 (Kennedy 2000). Authors at this time advocated regular mowing, rolling, weeding and obtaining good seed. The seed available would have been at best locally harvested landraces or ecotypes rather than bred varieties as we know them today. Clearly, the invention of the lawn mower eased the management demands of amenity grass land, increased the quality of these spaces, and was probably the driving force for a requirement to develop grasses to further optimise their quality.

Research into turfgrasses and their culture began in Michigan around 1880 and more formal research in Connecticut in 1890 and many states and Canada following suit during the next 30 years. In the UK the Board of Green Keeping Research was set up in 1929, funded by the Royal and Ancient, ultimately becoming the Sports Turf Research Institute in Bingley, West Yorkshire. Most of the early turfgrass research was driven by the efforts and monetary support of the golf interests in English-speaking countries and has rapidly expanded to include all sports in most countries.

2 Turfgrass Variety Development

Turfgrass breeding is a relatively new activity. The breeding of major crop species like cereals has been going on since the Neolithic revolution 10,000 years ago when man began to develop crop cultivation techniques. In contrast, improved varieties of turfgrass have only been developed in the last 40–50 years, often on the back of forage grass breeding developments. Turfgrass or forage grass species cannot be considered as truly domesticated in the sense that the major cereal crop species can. The latter have been developed with traits such as large seed size, seed retention, lack of dormancy, self-fertility, increased fertility, uniform flowering and seed ripening none of which forage grass varieties are fixed for. In the cereals this has resulted in genetic homogeneity and reproductive isolation from the wild progenitors. Forage and turfgrass varieties are genetically heterogeneous for these domestication traits and other agronomically important traits and are reproductively contiguous with wild populations. This has meant that a proportion of the forage/turf plant breeder's time is spent in maintaining a variety's performance over generations of multiplication which is undoubtedly eroded by genetic drift caused by inbuilt heterogeneity and susceptibility to cross contamination with wild relatives.

Most breeding systems for turfgrasses are based on a number of mother plants that have been selected on the basis of progeny family performance through one or more cycles of selection. These mother plants are poly-crossed together in isolation. The resulting populations are maintained through multiplication of a limited

number of generations towards certified seed ensuring that this certified seed maintains the characteristics of the original poly-crossed synthetic population. Certifiable seed is only produced from the early generations (pre-basic and basic crops) of multiplication, i.e. certified seed cannot be used to produce certifiable crops.

Unsurprisingly most variety improvements have been made on vegetative characteristics where, for example, valuable turfgrass genotypes have been found in old pastures where plant populations have become adapted to tolerating and surviving continuous close, heavy grazing by sheep for many years. The classic case is the selection of perennial ryegrass genotypes from 'Sheep Meadow' in Manhattan's Central Park by Rutgers University Plant Breeder, Dr. Reed Funk, in the 1960s.

Rutgers University's turfgrass breeding programme is still the basis of much of the turfgrass seed industry in the USA. Germplasm donation and royalty share agreements with commercial plant breeding companies, many of which are based in the Willamette Valley Region of Oregon, ensure that a steady stream of new turfgrass varieties are tested and commercialised throughout the world.

Northern European turfgrass breeding programmes are primarily run by commercial companies although some publicly funded research feeds into breeding programmes from research institutes such as the UK's Aberystwyth University Institute of Biological, Environmental and Rural Sciences formed in 2008 from a merger between the Institute of Grassland and Environmental Research and two University Departments.

Turfgrass varieties are primarily based on worldwide collections of ecotypes that have been subjected to a range of selection pressures that make them suitable for particular environments and turfgrass management regimes. Ground-breaking varieties such as the perennial ryegrass variety 'Manhattan' are then used as the basis of new varieties with further ecotype germplasm, with desirable additional characteristics such as increased disease resistance or abiotic stress tolerance, being introduced into the population followed by a recurrent selection process.

3 The Function of Amenity Grasses

The use of turfgrasses in the modern world fits into three major functional groups: Sport, landscaping and the provision of so-called ecosystem services. The first two are well-established functions, yet the potential of significant ecosystems service provision from extensive land areas covered by turfgrasses is only recent (for example, see Agros Associates 2004).

In the past, pastoral-type species such as perennial ryegrass *(Lolium perenne* L.), chewings fescue *(Festuca rubra* subsp. *commutata* Gaudin, syn. *Festuca nigrescens* Lam.) and browntop bent *(Agrostis capillaris* L. syn. A. *tenuis* Sibth.) were used on sports fields and recreational areas in temperate climates (Hickey and Hume 2003). These pastoral types would also have been used in landscaping situations.

Most modern turf species are now bred to meet specific amenity needs (see Table 1) and incorporate improved traits such as high shoot density, colour and fine texture. Differences in wear and mowing height tolerance amongst grass species

and varieties have also been shown to have dramatic effects on the appearance and durability of natural turf surfaces. Much of the work assessing the suitability of grass species and varieties as well as the overall quality of playing surfaces has been carried out by the STRI (Sports Turf Research Institute) (Canaway 1981, Gooding and Newell 1990, Newell 2001). Newell and Jones (1995) noted that the most wear-tolerant perennial ryegrass varieties for soccer were amongst the least wear-tolerant for tennis. It is therefore very important that grasses are tested under such management and wear treatments that simulate their intended use. This increase in knowledge of the function of turf species has led to huge progress in the development of varieties.

Large differences in football/rugby-type wear tolerance between varieties have been demonstrated (Canaway 1981) and various studies have demonstrated differences amongst grass species and varieties under abrasive wear (Newell and Wood 2000). These workers also reported on the performance of different grasses after prolonged close mowing, finding that the finer grasses (red fescues and bentgrasses) were the most attractive under close mowing but that coarser turfgrasses and in particular smooth-stalked meadow grass performed better when they were tested under close mowing and abrasive wear. Baker and Canaway (1993) have reviewed the literature relating to the playing quality of sports surfaces. They identified a number of components of playing quality which fall into two groups: (1) interactions between the ball and the surface; (2) interactions between the player and the surface. The importance of each group will depend upon the nature of the game in question.

Amenity grasses can also be grouped in relation to the intensity of culture required to maintain an appropriate surface, and the suitability of the species and variety to deliver under specific management practices. Grossi et al. (2004) identified these groups as

(a) Greens
(b) Sports turf
(c) Lawns – landscaping
(d) Functional – ecosystem services

Clearly, the choice of species and breeding objectives are strongly influenced by the intended end use, as illustrated by the comparison of landscape turf under shade and sports turf (Figure 1)

There is a growing realisation that turf has significant benefit in terms of ecosystem service provision beyond the traditional classification above. Furthermore these ecosystem advantages apply to all natural turf, to a greater or lesser extent.

3.1 Greens

For sports played on fine turf such as golf and bowls, the ball roll characteristics are of great importance. The effects of rolling resistance are referred to by players as the greens' speed, a faster green is more desirable. Fine-leaved species of *Agrostis* (*A.*

Table 1 List of species and their amenity uses (see crop specific chapters 10, 11, 15, and 16 for species description)

	Major uses			Minor uses			Niche		
Species	Greens	Sports pitches	Fairways/ lawns	Tennis/ cricket	Polo/racing	Low maintenance	Land reclamation	Shade	Drought
Lolium perenne		√√	√√	√√	√√				
Festuca arundinacea		√√			√	√			√√
Festuca rubra ssp. *rubra*			√		√	√	√		√
Festuca rubra ssp. *commutate*	√√			√					
Festuca rubra ssp. *trichophylla*	√√			√					
Festuca ovina			√				√√	√	√
Agrostis capillaris	√√		√√	√	√	√	√√		
Agrostis stolonifera	√√								
Agrostis canina	√√								
Deschampsia caespitosa		√					√√	√	√
Koeleria cristata						√	√	√	√
Poa pratensis		√√	√√		√	√		√	
Poa trivialis								√	

√ = used, √√ = predominant

Fig. 1 Landscape turf under deep shade (*left*) and soccer fields such as the Millennium Stadium in Cardiff, UK (*right*) are calling for largely different breeding objectives (Photos S. Duller)

capillaris, *A. stolonifera* and *A. canina*) and red fescues (*F. rubra* ssp. *commutata* and *F. rubra* ssp. *tricophylla*) are tolerant of close mowing and generally produce a close textured fine turf, whilst strong creeping red fescue (*F. rubra* ssp. *rubra*) has been proved to be unsuitable for mowing to 5 mm (Newell and Gooding 1990).

For lawn tennis and cricket wickets it is the ball bounce that is important. High-class lawn tennis courts, particularly those used for tournament play, are subjected to close mowing and intense abrasive-type wear, (Newell et al. 1996). Cricket wickets are a special case, prior to a match the turf is mown to ground level and the area rolled heavily to create a hard even surface to bowl on to, the quality of the soil and root zone being the key factors. Traditionally fine-leaved species were used for tennis and wickets because of the requirement for close mowing, however, improvements in mowing tolerance of ryegrasses through breeding has made it a popular choice.

Tolerance of close mowing for different grass species and varieties within species has been tested at the STRI for a number of years. This work is summarised in the annually published *Turfgrass Seed* booklet, the most recent being *Turfgrass Seed 2009* (STRI 2009). The aim is for as close textured and even playing surface as possible.

3.2 Sports Turf

Sports that involve a greater degree of player/pitch interaction, such as Soccer, Rugby, American football and field Hockey, require turf that is able to withstand greater wear. For cricket outfields the wear tolerance is less critical and frequently a mixture of ryegrass, fescue and bent grasses are used.

Sports turf quality is of fundamental importance to player safety and will also influence the players' enjoyment of a game. With Rugby player/surface interactions are the most significant playing quality attributes. Players are running and changing direction frequently, thus the amount of grip which the surface imparts to the player is important. Surface hardness is also relevant in Rugby because there is a

high degree of player/surface contact when falling or diving onto the turf. The significance of ball/surface interactions in Rugby is complicated by the characteristic shape of the ball which makes these interactions difficult to appreciate, unlike soccer and hockey where ball bounce and roll are also important playing quality attributes.

Early work on the playing quality of soccer pitches identified a series of reproducible tests and a sampling strategy which could be used in measuring playing quality (Winterbottom 1985). This led to the formulation of standards for the playing quality of natural turf soccer pitches (Bell and Holmes 1988).

Turf used for golf tees, fairways, cricket outfields, polo and racing can also be placed in a sports category due to its mowing height and wear type. Golf tees come under quite intense wear pressure all year round and are often sown with ryegrass-based mixtures. Fairways and cricket outfields are less intensively worn although there will be hot spots of wear in the bowlers run up and at popular entry/exit points on the fairway. The turf used is frequently a broader mixture of species and may include ryegrass, fescues, bent and/or smooth-stalked meadow grass. Polo and racing create slightly different conditions in that the wear type is often severe divoting, so turf strength and ability to recover quickly are key features.

3.3 Lawns and Landscaping

Lawn turf is a grass mown regularly to an even height, normally 15–30 mm, but shorter, approximately 10 mm for fine ornamental lawns. Lawns are used as landscaping features around homes, in parks, institutions, industries, schools and cemeteries. They can be purely ornamental or have recreational and functional aspects to their purpose. Natural grass provides a surface that absorbs light and heat, it has been proven by Devitt et al. (2007) and Williams and Pulley (2006) at Brigham Young University that natural turf maintains a consistently lower temperature in hot weather than artificial turf, concrete, asphalt and bare soil. It also filters rainfall and slows run-off, helping to reduce soil erosion.

Lawns are frequently the main feature of private and public gardens, whether they are ornamental, creating a foil for flower beds and as a design aspect, or for provision of a general recreational surface.

3.4 Functional – Ecosystem Services

Turf that is used for a purely functional purpose is usually kept under a very low-maintenance regime once initial establishment is achieved with no fertiliser and mowing once or twice a year. These situations include roadside verges, airfields, golf roughs, industrial site reclamation and ditch banks. Their purpose is to stabilise the soil, slow water run-off and in many situations provide wildlife habitat and help improve diversity.

The vast potential for adaptation of the grass family to a wide range of edaphic and climatic conditions means that they are highly suitable for carrying out a

range of functions beyond simply being used as ground cover. Beard and Green (1994) give a comprehensive review of the ecological and socio-economic benefits of turf. Below are a number of areas of ecosystem service provision that genetic improvement of non-forage grass species could have a significant impact upon.

3.4.1 Nutrient Dispersal and Cycling

Turf, in general, as a permanent ground cover provides a system for reducing nutrient run-off and increasing infiltration rates. Decreased run-off of phosphate and nitrogen have been documented as a result of turfgrass growth (for example, Linde and Watschke 1997). Perennial ryegrass forage grass breeding has resulted in selections for improved nitrogen use efficiency (Wilkins et al. 2000). Such genetic variation may be suitable to select for turfgrasses with an ability to perform at lower nutrient levels or to absorb excessive nutrient levels from soils.

3.4.2 Land Reclamation on Derelict and Contaminated Industrial Sites

Although grass species are not particularly good at accumulating pollutants and cannot therefore be used extensively for phytoamelioration (see Ebbs et al. 1997) they have proven ability, given increased fertility, to provide excellent vigorous cover of contaminated sites. Smith and Bradshaw (1979) demonstrated tolerance of a range of grass species to heavily contaminated mine sites in the UK and their surveys led to the development of three commercially available varieties of grass: *A. capillaris* varieties, 'Goginan' and 'Parys', tolerant of acid lead and zinc wastes and copper wastes, respectively, and a *F. rubra rubra* variety, 'Merlin', that is tolerant of calcareous lead and zinc wastes.

3.4.3 Carbon Sequestration and Climate Change Mitigation

Carbon sequestration in the soil profile (i.e. the relatively long-term storage of C in the 'stabilised' soil organic matter (SOM) fraction) is a significant climate change mitigation factor in the face of anthropogenic climate change induced by increased carbon emissions. It depends on a number of soil, plant, climate and management factors, and their interactions. Efforts to enhance carbon sequestration are justified by the declines in SOM reported, irrespective of soil type and land use, across England and Wales (Bellamy et al. 2005). Plants affect the quantity, quality and placement of C in the soil profile, through net primary production (i.e. the balance between photosynthesis and respiration), tissue composition (e.g. lignin content), litter production, root turnover, exudation and decomposition; all of which are subject to variation depending upon soil chemical, physical and microbiological conditions, climate and management. Long-term equilibrium levels of SOM are substantially higher in grassland soils, especially permanent pasture, compared with arable cropped soils. Soussana et al. (2004) calculated a figure of 25 t C ha^{-1} for the average difference in soil (0–30 cm) organic carbon stock between temperate

lowland cropland and pasture. Whilst ecological studies have demonstrated considerable variation in SOM under different types of grassland (i.e. high levels in acidic upland peat soils), the potential for exploiting inter- or intra-specific genetic variation amongst grasses for C deposition and sequestration in the soil has not been explored. Neither has there been any attempt to select and breed amenity or forage grass genotypes exhibiting enhanced C sequestration. Consequently, options for enhancing C storage in grassland systems have so far focussed on conversion from temporary to permanent grassland and alterations in N input.

3.4.4 Water Filtration and Purification

Reed bed systems (see Cooper 1999) have been used for many years for treatment of sewage. A number of grass species are used including *Phalaris* spp., *Phragmites communis*, and the systems work on the principal of the grass plants acting as a natural aeration system delivering oxygen to the water in which the plants are growing to encourage aerobic micro-organisms, associated with the roots and leaf litter, that are able to break down sewage and filter denitrified water that flows through the system (Gersberg et al. 1986).

4 Turfgrass Breeding Objectives

The overall objective of a turfgrass breeding programme is to produce a variety which, under turf management, is both visually appealing and fit for purpose. In addition it is paramount to the success of any grass variety that it is commercially sustainable in the long term. Along with turf performance this success depends on the variety's seed production potential. Selection procedures for seed yield need to be integrated into variety selection programmes.

Turfgrass evaluation for breeding (Figure 2) is a complex process involving many factors. The primary criteria will be the end purpose of the turf, but its quality will also depend on the time of year, the species present (including weeds and disease)

Fig. 2 Turfgrass evaluation of closely mown creeping bent grass at STRI, UK (*left*) and of perennial ryegrass under heavy wear at IBERS, UK (*right*) reflecting the intended use for golf greens and sports pitches, respectively (Photos S. Duller)

and soil quality, in addition, since many of the assessments are made subjectively, the skill and experience of the person carrying out the assessments.

Mowing is fundamental to turfgrass management and has a major influence on turfgrass quality, function and playability. Although turfgrasses tolerate mowing, they differ considerably in their growth and stress tolerance between mown and unmown plants. Mowing height may be manipulated along with frequency of cutting to improve turfgrass quality and function.

4.1 Growth Characteristics

There are several aspects of growth habit: angle of leaves, upright or prostrate stems and distance between internodes on the stem. These features affect the tolerance of the species or variety to mowing and subsequently the smoothness of the turf. The way the leaves and stems lie determines the presence or extent of 'grain' within the turf surface. An upright habit will tend to create less graininess than a species or variety that is prostrate or creeping. The texture of the turf is also affected by the fineness of leaf. Narrow leaves are desirable in fine turf applications (bowls and golf) to provide a uniform and fast playing surface. Leaf width varies greatly between species and is also affected by cutting height and soil nutrition. *Agrostis* species and *Poa annua* leaf width can be reduced by 50% by lowering the cutting height from 38 to 8 mm (Beard 1973). There is little variation in leaf width within the fine leaf species of fescue. However, within ryegrass and smooth-stalked meadow grass there is considerable variation in leaf width between varieties.

Another factor affecting the quality of a sward is cleanness of cut. Differences in the fibre content of the leaf affect the cleanness of cutting, high fibre content will leave a ragged cut surface which will bleach more readily affecting the appearance. Poor cleanness of cut whether through high fibre or silica content or blunt (or inappropriate) machinery will increase the surface area of damaged leaf tissue providing a favourable entry point for fungal diseases. Frequent mowing also enhances disease development by providing regular wounds at the leaf tips.

There is variation in grass growth throughout the year due to day length and temperature. Some temperate species display very little dormancy through the winter and keep growing when conditions allow or show early spring growth. The rate of re-growth after mowing also varies and is a critical factor as mowing accounts for the majority of the cost of maintaining amenity grass. Counter to this, fast re-growth may also help to ensure faster recovery from wear in some situations. The mowing frequency can be as important as the cutting height and longer intervals between mowing may improve the overall vigour of the sward, providing that not more than 40% of the leaf area is removed at any one mowing (Madison 1960).

4.2 Uniformity/Visual Merit

A high-quality turf should be uniform in appearance. Thin areas, disease, irregular re-growth and poor texture all detract from a sward. Turf needs to be fit for purpose,

and alongside the provision of a safe and suitable surface (especially for sports pitches) the high level of televised games and matches have driven an increased desire for swards with a high level of visual merit. Visual merit is the overall measure of the suitability of the sward for its potential use and will incorporate sward density, texture, leaf width, disease and weed incidence, growth habit and colour. All these factors need to be considered when selecting plants and populations for variety development.

4.3 Density

Shoot density is one of the most important aspects of quality. In most applications of amenity grass a dense sward is required, especially when a uniform and true playing surface is required. The number of shoots per unit area is assessed to give a measure of sward density of the sown species. Visual quality ratings are positively correlated to shoot density (Bruneau et al. 2000). A high shoot density is important to help compete against weed invasion and to protect the soil surface from wear. There are large differences in shoot density between species and between varieties within species (Newell and Gooding 1990). Wear, time of year and cutting height will also affect shoot density. Reducing mowing heights and increasing mowing frequency enhance playing conditions (up to a point). Suboptimal mowing heights reduce leaf widths, shoot size and improve texture but they also reduce leaf areas, impeding the turfs ability to capture sunlight and synthesise carbohydrates. The associated increase in shoot growth reduces net carbohydrate reserves and root production, so stress tolerance is reduced. Greens mowed closely (3–2.5 mm) for enhanced putting speed require more frequent watering, fertilising and pesticide applications to maintain the desired quality than those mowed only slightly higher (4–5 mm) obviously this also reduces the costs (Shearman 1985).

4.4 Turf Colour

One of the most visible turf characteristics is its colour, which can convey much information as to its physiological well-being and its nutritional status and so is a useful measure of turf performance. Turf breeders and assessors need to be able to objectively measure turf colour and colour change, in order to determine varietal/breeding line differences and to relate these differences to turf performance. Perceived colour results from the wavelength of visible light reflected from a given object. The colour of turfgrass varieties grown under optimal conditions can be measured in precise numerical terms using the parameters hue angle (H°), chroma (C^*) and value (L^*). Colour scores are determined by the physiological and physical attributes of individual leaves and by the spatial arrangement and proportion of component parts of a grass sward. The onset of less favourable growing conditions for a turf can result in reduced plant growth rates and increased rates of senescence, which are indicated by unacceptable colour changes. There are varietal differences

in the rates of colour change indicating that unacceptable colour changes can be minimised, not only by good cultural practice but also by informed varietal choice. Generally, hue angle is reduced linearly during senescence and so reduction, in a turf situation, indicates an increased proportion of senescent grass material. Changes in value and chroma, however, follow parabolic curves and so cannot be used to indicate increased levels of senescence and discoloration. Maintenance of H° provides an indicator of a breeder's success in integrating a range of resistance and tolerance in a grass variety, which may be expressed as a response to stress conditions encountered during its lifetime. Colour stability (McMichael and Camlin 1994) is an important characteristic on which variety discrimination tests can be based. There are differences in colour between varieties but a significant proportion of the total variation can be assigned to variety × environment interaction including seasonal differences. In general, greener (high H°) and darker, duller (low b∗) turfgrasses are preferred by the end user (Thorogood 1995, 1996). This is especially the case in the USA.

4.5 Wear Tolerance and Turf Tensile Strength

As a major function of amenity grass is to act as a permanent, resilient surface subject to, in many cases considerable, impact from mechanical damage by machinery and human contact, tolerance to wear and abrasion is essential.

Wear on turfgrass involves damage and removal of biomass and may involve gradual removal by abrasion or immediate removal of divots. Therefore a sward's ability to withstand wear and also to recover from wear are important tolerance strategies.

One of the difficulties in measuring wear tolerance of turfgrass is being able to artificially simulate real wear conditions on playing fields (see Canaway 1976b). Nevertheless, the relative wear tolerance of different grass species has been evaluated empirically and perennial ryegrass, smooth-stalked meadow grass and tall fescue are considered to be the most wear tolerant (as reviewed by Canaway 1975a). In addition, within species variety differences in wear tolerance have been observed (see Canaway 1975a). Turfgrass variety trials and, in many cases, breeder's trials are routinely subjected to artificial wear using mechanical devices such as studded rollers. One of the pioneering devices for testing turf wear and empirically assessing the relative wear tolerance of different sports surfaces, the differential slip wear machine was developed at the Sports Turf Research Institute (Canaway 1976a). This machine imparts wear from two studded rollers which turn at different speeds thus imparting horizontal as well as vertical forces on the turf surface. A major concern with any testing system is that the experimental system replicates real-life scenarios. Undoubtedly, varietal differences have been observed in turfgrass wear trials yet how representative these differences are in actual practice is open to question.

An understanding of the tolerance mechanisms employed by grasses would enable breeders to select for particular characteristics. However, the types of wear

and the environmental conditions under which wear occurs are diverse and the response of plants will vary depending on these factors. The mechanisms of wear tolerance are extremely complex with a range of underlying physiological, anatomical and morphological components. Shearman and Beard (1975) found significant correlation between species differences in wear tolerance with leaf tensile strength and leaf width characteristics of individual plants. However, within species differences for these characteristics are unlikely to result in significant differences in wear tolerance. More likely, wear tolerance will be affected by the turf sward structure, and total biomass before wear begins has been found to be a good indicator of biomass that remains after a period of wear in perennial ryegrass (Ellis 1981).

Recuperative ability will also be important, and, in cases where large divots of turf are completely removed, the ability of plants to spread, by rhizomes or stolons, into the bare areas is critical. The ability of a turf to withstand and recover from wear will also be dependent on environmental constraints and, although summer wear tolerance is found to be associated with larger pre-wear biomass, wear tolerance in the winter periods, especially when rainfall is high and temperatures are low, is much more dependent on a swards ability to continue growing at low temperatures in saturated and compacted soils. Annual bluegrass has been found to be particularly wear tolerant in highly compacted soils and often becomes the dominant species on highly worn areas of sports pitches.

In sports where there is a crucial maximum cutting height above which playing quality is unacceptable, one possibility that plant breeding presents is to increase biomass at the playing height by selection of appropriate varieties. There are many varieties which show variation for biomass at a given cutting height. Biomass is significantly and positively correlated with shoot density and shoot density with wear tolerance (Figure 3A and B), so it is possible to produce varieties which combine fine texture with wear tolerance.

But the correlation only accounts for a small proportion of the variation so there is a danger that in selecting grass for wear, low shoot densities may result. Conversely by selecting for high shoot densities, wear tolerance may be compromised.

The tensile strength of turf is important especially in applications where divoting is a problem such as sports fields, golf tees, race courses and polo grounds. The Sports Turf Research Institute has been at the forefront of developing equipment for the empirical testing of the tensile strength of turf in situ (Canaway 1975b).

Tensile strength of harvested turf is also critical in the turf industry where turf needs to be strong enough to be lifted, rolled, transported and re-laid. Ross et al. (1991) found that turf strength was related to a number of root architecture characteristics when comparing different species of turfgrass and they developed indices of these characteristics to maximise turf strength.

4.6 Growth at Low Light Intensity

Increasingly sports stadia are becoming larger in terms of audience capacity and, by necessity, more enclosed as stand height increases (Baker 1995a). There is

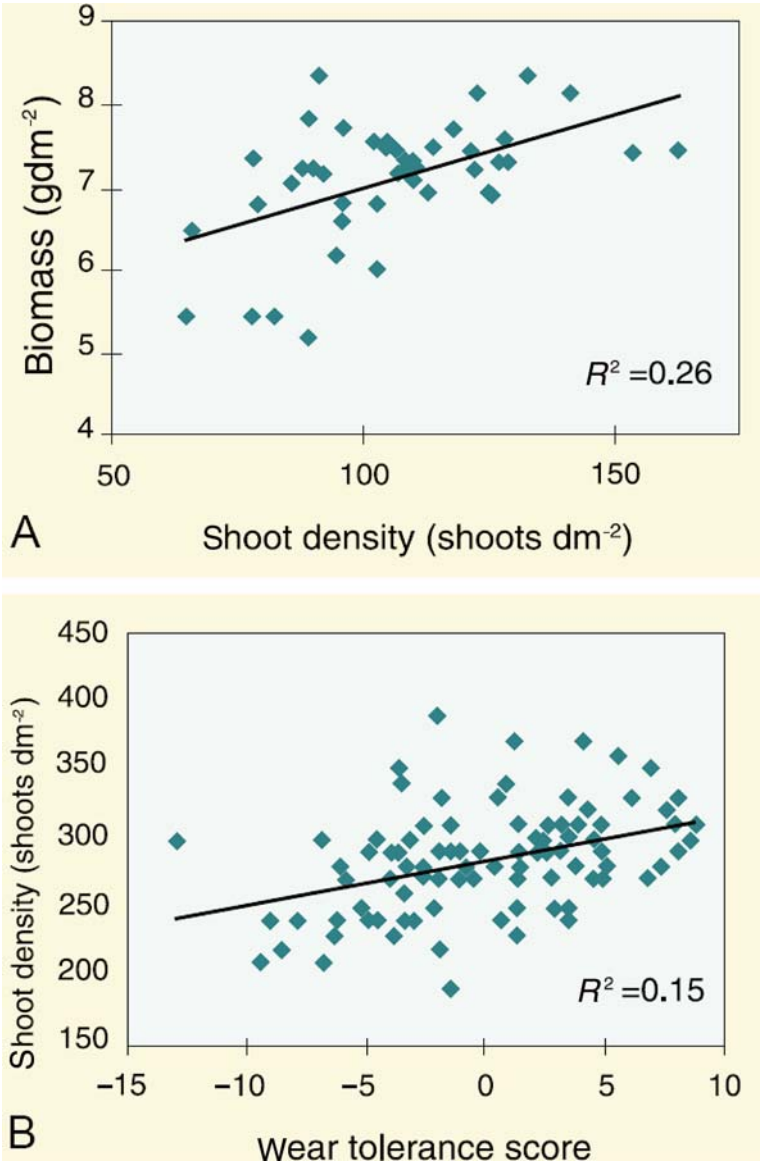

Fig. 3 Relationship between shoot density and (**A**) plant biomass below cutting height (**B**) wear tolerance rating (Thorogood pers. com.)

also a trend towards closing roofs to improve the versatility of the space. This has a hugely detrimental effect on the quality of the playing surface as the conditions within are deeply shaded and lack air movement (Baker 1995b, Newell et al. 1999). Light levels are frequently so low that respiration exceeds the rate of

photosynthesis and the grass becomes weakened and dies. Combined with the other stresses associated with sports pressures, this produces an extremely hostile environment for plant growth. Maintenance of these surfaces is reduced to damage limitation, made more difficult by the ever increasing economic demand for a maximum number of fixtures to be staged within the shortest possible time. Although variety development work is focussing on improving the shade tolerance of varieties, and it has been shown (Tegg and Lane 2004) that the turfgrass species *Poa supina* Schrad. and *Festuca arundinacea* Schreb. had greater shade tolerance than other species tested, the mechanisms that plants use to cope in shaded situations tend to work against the creation of good turf. For example, leaf growth tends to be long, open and upright in a shaded situation, and species that are naturally adapted to shade tend to display this type of habit. Pitch management plays a key role, under such biologically challenging growing conditions. Where match fixtures are restricted, light levels can be optimised by appropriate stadium design and artificial lighting, and a strategy of pitch renewal by re-turfing on a regular basis during the main playing season can be implemented.

4.7 Tolerance of Other Abiotic Stresses

Turfgrasses are subjected to a plethora of abiotic stresses, such as extremes of temperature and water stress, yet there is no real evidence of varietal tolerance improvement in national turfgrass evaluations. Up until recently, the solution to such problems has been solved through appropriate management practices but current concerns over sustainable practice may well see a greater incentive for breeders to target a genetic solution. The stresses often involve several interacting factors that require a whole range of plant strategies, presumably under complex genetic control, before wide-ranging tolerance can be achieved. The genetic control of tolerance to abiotic stress in grass species is gradually being elucidated and will ultimately lead to the production of improved varieties. The presence of endophytic fungi which form a symbiosis with many grass species has also been associated with abiotic stress tolerance improvement.

4.8 Resistance to Pests and Diseases

Densely populated turfgrass communities provide a perfect micro-climate for the development of a range of pests (Potter and Braman 1991) and diseases (Tani and Beard 1997). However, as with abiotic stress tolerance, consistently and widely resistant varieties have not been so far largely developed. Integrated management of pests and diseases will be required, with biological control and plant resistance strategies playing a key role in ensuring sustainable practice. Endophytic fungi may also play a key role in controlling a range of pests and diseases.

5 Progress and Future Objectives in Turfgrass Breeding

Since the initiation of targeted turfgrass breeding programmes and the replacement of grasses selected on the basis of their agricultural importance with bespoke turfgrass varieties, considerable improvements have been made in terms of ground cover, shoot density, leaf texture and turf quality for all grass species currently used for amenity purposes. This is demonstrated by the performance of modern grass varieties compared to older control varieties that are included for reference purposes in the National Turfgrass Evaluation Programme (www.ntep.org) which co-ordinates a USA-wide variety testing system, the most extensive multi-site turfgrass trialling system in the world. Similarly, variety improvements are shown by the trial results of the Sports Turf Research Institute (STRI) in the UK in the last 25 years (Table 2). For a number of traits there is no indication of variety improvement over this period of time. Moreover, in parallel with the agricultural green revolution, much of the improvement has been dependent on the use of high levels of artificial chemical inputs, such as inorganic fertilisers and synthetic pesticides, for improving grass growth and preventing weeds, pests and diseases in the quest for the perfect green surface. Furthermore, there are considerable costs involved in maintaining turf surfaces, mainly from the need for frequent mowing, but also through other management practices such as irrigation, drainage, aeration and dethatching required to maintain an optimum turf surface. Many of these practices, being dependent on finite fossil fuel resources, through chemical manufacture and transport and through fuel costs for running machinery, are unsustainable. Furthermore, the burning of fossil fuels is the primary cause of anthropogenic climate change induced by carbon dioxide production. Awareness of environmental sensitivities to excessive fertiliser use is increasing with, for example, the implementation of the 1991 European Union Nitrates Directive and the designation of nitrate vulnerable zones (NVZs). Pesticide legislation is also restricting their use as more toxicological data becomes available:

Table 2 Correlation coefficients between year of first registration and perennial ryegrass cultivar performnce for traits measured in STRI trials (Cultivars registered between 1983 and 2007)*=significant at P<0.05, **=significant at P<0.01, ***=significant at P<0.001

Trial type	Trait	R^2	df	Sig. level
Winter wear trial	Shoot density	0.304	108	***
Lawn trial	Shoot density	0.273	110	***
Lawn trial	Visual merit	0.223	110	***
Winter wear trial	Live ground cover	0.188	108	***
Winter wear trial	Visual merit	0.185	108	***
Winter wear trial	Leaf fineness	0.139	108	***
Lawn trial	Winter green	0.126	110	***
Winter wear trial	Recovery from wear	0.106	108	***
Lawn trial	Slow re-growth	0.104	110	***
Lawn trial	Leaf fineness	0.070	110	**
Winter wear trial	Summer green	0.053	108	*
Lawn trial	Summer green	0.051	110	*

manufacturers need to submit new data which are expensive to collate and decrease the viability of producing new products. Crop-specific labelling requirements will further restrict use of pesticides.

There are therefore considerable pressures forcing the turfgrass industry, like its agricultural counterpart, to be more sustainable and sensitive to environmental and human health needs.

For economic reasons turfgrass breeders have traditionally targeted traits such as slow re-growth that reduce mowing costs and significant progress has been made since the use of primarily agricultural grasses 40–50 years ago and there is a significant positive correlation between the year of variety registration and slow re-growth score in STRI trials (see Table 2).

Resistance to pests and diseases and nutrient use efficiency have always been useful targets for reducing input costs, yet progress to date has been relatively slow. A restriction on chemical use and the need for climate change mitigation mean that these traits will become primary breeding targets. Climate change will inevitably lead to both abiotic and biotic stresses and resistance and tolerance to these will also remain essential breeding targets.

There are an overwhelming number of reasons why these traits have been relatively difficult to enhance through breeding. It may be that the genetic variation on which to select is simply not present in existing germplasm; if present, genetic control is by many genes which need to be present in specific combinations; trait expression is influenced by the particular environmental parameters prevalent at specific locations. This is exemplified by often poor correlations between sites for recorded in the NTEP perennial ryegrass trials for the period 2000–2003 (Table 3).

The lack of variety rank correlation between the two (Quebec and Kansas) sites for leaf wilting (drought) ($R = 0.10$, 94 degrees of freedom), and between the two sites (Nebraska and Arkansas) where winter kill was measured ($R = –0.09$, 94 degrees of freedom), is presumably due to the fact that different environmental parameters for these traits were prevalent at only one or the other of the sites. For disease resistance, the situation is further challenged by the adaptive nature of the pathogen where new races can quickly evolve and overcome variety resistance. A combination of low heritability, environment × genotype interactions and pathogen evolution is no doubt responsible for the lack of apparent progress in breeding for stable across-site resistance to important turfgrass pathogens.

Many of the sports environments that turfgrasses are used in are artificial, typically creating, and often combining, a large number of biotic and abiotic stress factors.

Turfgrass breeders are continually seeking new sources of resistance to these stresses through extensive plant collection of natural ecotypes subjected to specific selection pressures and have introgressed these ecotypes into their breeding pools in order to extend the range of variation for traits suited to lowering the maintenance costs and increasing the sustainability of turfgrass cultivation. In some cases this has involved introgressing germplasm from other species. For example, much research has been directed towards introgression of *Festuca pratensis* and *F. arundinacea* germplasm into *L. perenne* (see Chapter 12, *Festulolium*).

Table 3 Correlation coefficients between trial sites for perennial ryegrass cultivar scores for various disease resistances in NTEP trials (2000–2003)

(a) Brown Patch (causal organism, *Rhizoctonia solani* Kuhn)				
	NE	PA		
IL	**0.072^{NS}**	0.262**		
NE		0.270**		
(b) Dollar Spot (causal organism, *Sclerotinia homoeocarpa*) F.T. Bennett				
	MI	NJ1	NJ2	RI
KS	**0.055^{NS}**	0.194*	0.206*	**0.013^{NS}**
MI		0.402***	0.324***	**0.001^{NS}**
NJ1			0.469***	**0.001^{NS}**
NJ2				**0.173^{NS}**
(c) Gray Leaf Spot (causal organism, *Pyricularia grisea*) (Cooke) Sacc.				
	MD	NJ1		
IL2	**–0.089^{NS}**	**0.080^{NS}**		
MD		0.369***		
(d) Red Thread (causal organism, *Laetisaria fuciformis*) (McAlpine) Burds.				
	VA	WA		
NJ	0.223*	0.385***		
VA		**0.096^{NS}**		

Abbreviations: KS – Kansas; MA – Massachusetts; PA – Pennsylvania; MI – Michigan; NJ 1 and 2; New Jersey sites 1 and 2; RI – Rhode Island; MD – Maryland; NY – New York; VA – Virginia; WA – Wisconsin NS = not significant, *=significant at P<0.05, **=significant at P<0.01, ***=significant at P<0.001. All correlation coefficients with 133 degrees of freedom.

An alternative to improving the grass species themselves, the grass endophytic fungi of the genus *Neotyphodium* that inhabit the shoot bases of many grass plants have provided another potential tool in the defence against a range of biotic and abiotic stresses. One of the first potentially useful discoveries was the association between an endophytic fungus (*Neotyphodium lolii*) and resistance to sod webworm (*Crambus* spp.) (Funk et al. 1983). The presence of potent insecticidal alkaloids, particularly peramines and, in some *Neotyphodium* species, lolines, produced during the symbiotic grass–fungus association, provides a natural defence against potential pathogens. Although the mechanisms are not fully understood, endophytes have also been shown to confer tolerance to drought and mineral stresses (Malinowski and Belesky 2000). Schardl et al. (2004) have produced a comprehensive review of the documented effects of grass–fungal endophyte symbionts on insect, nematode, fungal and plant growth, physiology and behaviour. Of great advantage to the breeder is that the fungus is transmitted vertically through seed so any of its properties are effectively maternally inherited through seed generations and can be easily maintained over generations of subsequent seed multiplication.

6 The Impact of Biotechnology on Turfgrass Breeding

As with all other grass breeding programmes, in the future, a more directed approach is required if further advances in turfgrass performance are to be made from

those achieved through conventional means. Modern DNA-based molecular marker technology has enabled a thorough understanding of the genetic control of plant processes and has also given rise to the potential to select directly for genes underlying the expression of many traits of interest to plant breeders (see Chapter 4).

Genetic linkage maps, which provide the basis for associating agronomic traits with specific DNA sequences, have been produced for a number of forage species (see Chapters 10 and 11). Specific to applications in turfgrass breeding, maps for creeping bentgrass (*A. stolonifera* L.) (Chakraborty et al. 2005), colonial bentgrass (*A. capillaris* L.) (Rotter et al. 2009), Texas bluegrass (*Poa arachnifera* L.) (Renganayaki et al. 2005) and Kentucky bluegrass (*Poa pratensis* L.) (Albertini et al. 2003) have added to the map information available in the grasses. Marker-saturated genetic maps provide the genetic framework necessary for identifying and selecting for individual genetic components or quantitative trait loci (QTL) associated with important traits (see Chapter 4) and QTL mapping has been conducted in a number of turfgrass species. As with the genetic mapping studies, the ryegrasses (Chapter 10) have received the most attention in QTL analyses. Of direct relevance to turfgrass-breeding objectives, QTL have been identified for winter hardiness-associated traits (Yamada et al. 2004), crown rust (*Puccinia coronata* f. sp. *lolii*) resistance (Muylle et al. 2005, Studer et al. 2007) and resistance to gray leaf spot (*Magnaporthe grisea*) (Curley et al. 2005). Additionally, traits influencing flowering and seed production are also important for breeding ryegrasses whether for use as turf or forage. QTLs have been identified for heading date (Armstead et al. 2004; King et al. 2008) and seed set (Armstead et al. 2008).

Fewer QTL studies have been completed in the other cool-season turfgrass species. Research is currently being conducted to determine QTL associated with brown patch resistance (*Rhizoctonia solani* Kühn), in tall fescue (unpublished results). In creeping bentgrass, a single major QTL for dollar spot resistance (*Sclerotinia homoeocarpa* F. T. Bennett) was identified by Chakraborty et al. (2006). Additional studies in creeping bentgrass have identified QTL for dollar spot resistance, drought tolerance and heat tolerance (Bonos, pers. communication). In colonial bentgrass, Rotter et al. (2009) identified regions of the colonial bentgrass genome potentially involved in dollar spot resistance. In Texas bluegrass, QTL have been identified in association with the dioecy locus on the paternal map (Renganayaki et al. 2005).

Transgenic plants have been obtained for the majority of turfgrass species (reviewed by Fei 2008) including creeping bentgrass (Luo et al. 2004), tall fescues (Dong and Qu 2005), Kentucky bluegrass (Gao et al. 2006), perennial ryegrass (Altpeter et al. 2000; Wu et al. 2005), red fescue (Altpeter and Xu 2000) and colonial bentgrass (Chai et al. 2004).

Transgenic approaches have been utilised in an attempt to improve disease resistance in creeping bentgrass (Dai et al. 2003, Zhenfei et al. 2003), ryegrass (Takahashi et al. 2005) and tall fescue (Dong et al. 2007). Although transgenic turfgrasses may show improved resistance or delayed symptomatology they have not yet resulted in a commercial variety of turfgrass. Transgenic approaches have also been utilised in an attempt to improve abiotic stress tolerance in tall fescue (Lee et al. 2007, Zhao et al. 2007) and creeping bentgrass (Fu et al. 2007, Xing

et al. 2007). This approach will most likely expand in the future as more sequence and functional genomic information will be used to identify genes that can be incorporated into turfgrasses through transformation.

To date, the most successful transgenic application has been for herbicide resistance (Hartman et al. 1994, Wang et al. 2003). The closest product to commercial production is Roundup Ready® creeping bentgrass researched and developed by The Scotts Company in the USA. Roundup®-tolerance in the Roundup Ready® creeping bentgrass is conferred by a gene (*cp4 epsps*) from *Agrobacterium* sp. strain CP4 that produces a version of the 5-enolpyruvylshikimate phosphate synthase (EPSPS) with reduced affinity for glyphosate (Fei 2008, Padgette et al. 1996). Fei and Nelson (2004) assessed the potential risk of transgenic creeping bentgrass and found that none of the transgenic lines examined were significantly different from the respective non-transformed tissue culture lines for seed production characteristics. However, Wartud et al. (2004) provided clear evidence for pollen mediated gene flow of the *cp4 epsps* gene to wild bentgrass plants at the landscape level. Nevertheless, the Roundup Ready® creeping bentgrass developed by the Scotts Company is currently under review by the Animal and Plant Health Inspection Services of the United States Department of Agriculture (USDA – APHIS) (Fei 2008) and may be deregulated in the future.

7 Conclusion

Classical turfgrass breeding efforts will continue but manipulation of complex traits that modern turfgrass managers require will be enhanced by advances in molecular biology including gene mapping, comparative genomics, transgenic approaches, sequencing and functional genomics. The combination of techniques should result in improved turfgrass varieties which moreover address current demands to mitigate or adapt to climate change, to be adapted to a wide range of climatic and edaphic factors and also can be managed more sustainably without recourse to excessive demands on finite global resources such as potable water and fossil fuel-based chemical inputs.

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Breeding for Grass Seed Yield

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1 Introduction

Seed yield is a trait of major interest for forage and turf grass species and has received increasing attention since seed multiplication is economically relevant for novel grass cultivars to compete commercially.

Although seed yield is a complex trait and affected by agricultural practices as well as environmental factors, traits related to seed production reveal considerable genetic variation, prerequisite for improvement by direct or indirect selection. This chapter first reports on the biological and physiological basics of the grass reproduction system, then highlights important aspects and components affecting seed yield, and finally discusses the potential of plant breeding to sustainably improve seed yield in forage and turf grasses. Aspects of forage legume seed production are dealt with in the respective crop-specific chapters.

2 Biological Properties

Siphonogamy, the delivery of immotile gamete cells via a pollen tube to the archegonia, is the key mechanism allowing flowering plants (angiosperms) to carry out sexual reproduction.

The most commonly occurring mode of reproduction in forage and turf grasses is cross-fertilization, where male and female sex cells fuse from different individuals of the same species. In outcrossing grass species, cross-fertilization is maintained by a genetic mechanism, referred to as self-incompatibility (SI). SI is known to be controlled gametophytically by two multiallelic and independent loci, S and Z (Cornish et al. 1979). The recombination of genetic material from two different individuals maintains genetic variability, thereby affecting population structure, diversification potential, and the capacity to adapt to environmental change. However, SI is not

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always fully effective and variations in self-pollination rates have been observed between and within grass species.

Other forms of sexual reproduction such as apomixis (i.e., clonal seed production) have been described in Kentucky bluegrass (*Poa pratensis* L., see Chapter 15, Section 1.2). Apomixis allows the efficient fixation of multigenic traits over successive generations and is thus considered a plant breeding tool of major interest.

2.1 Flowering Biology

The inflorescences of Poaceae are, characteristically for each species, arranged in spikes or panicles made up of a number of spikelets, each having one or more hermaphroditic florets (Figure 1). Pollination is always anemophilous. The fruit of most forage and turf grasses is a caryopsis, meaning that the seed coat is fused to the fruit wall.

The initiation and coordination of flowering is essential for effective pollination, particularly in outcrossing grass species, which are exposed to temporal differences in head emergence, anther exertion, and pollen release of individual plants. Most temperate grasses have a dual requirement for flower induction (Heide 1994). The primary induction called vernalization proceeds in short days and/or low temperatures and the fulfillment of these requirements is characterized by the shoot apex being initiated for flower induction. Furthermore, the induced apex requires a secondary induction period under long day condition, a process hastened by high temperature, before the tiller will start to elongate and eventually develop into a flowering tiller. The underlying complex genetic processes of flower induction

Fig. 1 Flowering spikelets of perennial ryegrass (Photo H. B. Rasmussen)

reveal substantial variation within and between grass species and are of particular interest when breeding new cultivars adapted to different climates.

To date, two genes controlling flowering time were described in perennial ryegrass (*Lolium perenne* L.). *LpVRN1* was identified based on DNA sequence homology to *TmVRN1* of *Triticum monococcum* and is located on ryegrass chromosome 4 (Jensen et al. 2005; Andersen et al. 2006). Furthermore, a CO-like gene and putative *Hd1*-orthologue from rice (*Oryza sativa*), referred to as *LpCO*, were reported to affect vernalization response in perennial ryegrass (Martin et al. 2004; Armstead et al. 2005).

2.2 Pollination and Fertilization

In Poaceae, pollen is released from the anther as a dehydrated microspore containing the male gametophyte. The degree of pollen dehydration varies considerably between species, but the water content of pollen at anthesis is usually within the range of 15–35% of fresh weight (Dumas and Gaude 1982). Pollen is dispersed abiotically by wind. Generally, pollen dispersal corresponds to a two-dimensional distribution of a pollen cloud that dilutes rapidly with increasing distance, best described by an inverse quadratic function of inter-plant distance (Cunliffe et al. 2004).

Angiosperm stigmas can be assigned to two major groups, wet and dry stigmas, depending on whether or not they possess a surface secretion. Forage and turf grasses exhibit a dry stigma type, where pollen capture and adhesion to the stigma is largely a function of the pollen wall and exhibits a high degree of species specificity (Hiscock and Allen 2008).

Hydration of pollen on the stigma surface is a highly regulated process. In dry stigma species, pollen tubes secrete hydrolytic enzymes such as cutinases to breach a continuous cuticle during stigma penetration. Regulated hydration of pollen is clearly an essential element of normal pollen development on stigmas.

Once the cuticle has been breached, the pollen tube grows within the cell wall of papilla cells toward the base, where it emerges and continues to grow intercellularly toward the ovary. Several pollen-associated enzymes appear to be involved in these processes, most notably pectin-, cellulose-, and hemicellulose-degrading enzymes. Pollen tube growth is guided by various chemical components (Palanivelu et al. 2003). Some of these physiological mechanisms are highly sensitive to various environmental factors such as temperature, humidity, or salinity.

2.3 Seed Set and Development

The term seed set describes the early growth of the embryo and endosperm following successful fertilization. Plants generally produce more ovules that are available for fertilization than mature seeds. There are a number of reasons why a floret may

fail to set a viable seed. The florets may not be successfully pollinated because mature stigmas do not get viable pollen at the right time, possibly due to inhomogeneous flowering times or early lodging. Moreover, fertilization may fail if deposited pollen is not compatible due to SI. Early zygote development is highly sensitive to morphological and/or environmental disruptions, finally leading to abortion of the seed. Normally, ovule degeneration may occur soon after fertilization and only few seeds are aborted later than 10 days after anthesis (Elgersma and Sniezko 1988).

Embryo formation, or embryogenesis, usually occurs within a few hours after fertilization. The first cell division, being highly susceptible to environmental stresses, cleaves the zygote into two daughter cells. The following rapid cell division provides preliminary structures of the primary body parts, such as coleoptile, coleorhizae, and scutellum. Subsequent seed development can generally be divided into three phases. The initial growth stage is characterized by a rapid increase in seed weight and high seed moisture content. At this stage, i.e., the first 10 days after pollination, the seed is not viable. In the second phase, during 10–14 days, the seed reserves accumulate and a threefold increase in seed dry weight is observed. The seeds attain full viability. In the third phase, the ripening stage, the amount of dry matter is almost constant, but the total moisture content, fresh weight, and percentage of moisture decrease. In a fully developed grass seed, lemma, and palea consist of about 25%, the caryopsis of about 75% of the total dry matter, depending on the species. Dry matter accumulates for 3–4 weeks in most species, implying a daily dry matter increase of 3–4%. Climatic conditions influence seed development and ripening, hence the increase of fresh and dry matter is higher at low temperatures in early seed development and the ripening process is shorter at high temperatures.

3 Seed Yield Components

Seed yield is affected by several yield components and reflects the interaction between the seed yield potential (e.g., number of reproductive tillers, number of spikelets/florets per reproductive tiller), the utilization of the potential (e.g., seed set, seed weight), and the realization of the seed yield potential defined as the number of florets forming a saleable seed. The realization of the seed yield potential is affected by seed retention and other traits associated with yield loss during the harvest and post-harvest processes.

3.1 Reproductive Tillers

Flower induction requirements vary among grass species and between cultivars within each species according to origin of germplasm. Red fescue (*Festuca rubra* L.) requires 12–20 weeks of vernalization, whereas the requirement of perennial ryegrass (*L. perenne* L.) is fulfilled after only 3–8 weeks. From experiments

with six European perennial ryegrass cultivars, it was shown that the Mediterranean cultivar 'Veyo' had a requirement of 0–3 weeks, whereas the Scandinavian cultivar 'Falster' needed 7–8 weeks to fulfill primary induction (Aamlid et al. 2000). The same plant material was used to establish a perennial ryegrass F2 mapping population for the characterization of the genetic variability for vernalization requirement available between 'Veyo' and 'Falster' (Jensen et al. 2005). The detected quantitative trait loci associated with date to heading lead to the isolation and cloning of the *LpVrn1* gene involved in vernalization response of perennial ryegrass. For the number of reproductive tillers, however, the same mapping population revealed a low heritability ($h^2 = 0.56$) and no consistent genetic effect, when assessed on single spaced plants under constant glasshouse conditions (Studer et al. 2008). This is in accordance with other genetic studies reporting highly significant genotype × environment interactions for this trait (Fang et al. 2004; Ergon et al. 2006).

Tiller development influences transition rate from vegetative to reproductive growth with large tillers being more successful than smaller tillers (Boelt 1999; Meijer 1987). The effect varies among species with perennial ryegrass having a transition interval of 98–85%, in contrast to Kentucky bluegrass with 68–48% of autumn-produced tillers becoming reproductive. Flower induction stimuli may be transferred from mother to daughter tillers; however, still tillers emerging before or during primary induction have the highest chance of becoming reproductive (Havstad et al. 2004).

The number of reproductive tillers per unit area is an important component in establishing the seed yield potential; however, its importance varies among grass species. In slow-establishing species or species with a high vernalization requirement tiller number and tiller size in autumn are important factors in maintaining a high yield potential in first-year seed crops. The number of reproductive tillers per unit area is of low heritability.

3.2 Spikelets and Florets per Inflorescence

The total number of spikelets and florets per inflorescence depends on the number of primary branches and on the number of florets produced per primary branch. While there is only one spikelet per primary branch in ryegrass, basal branches of panicle grasses usually develop considerably more spikelets and florets than terminal ones. It is believed that the number of primary branches increases with apex size and it has been found that tillers developed first have more primary branches and more florets per primary branch than tillers developed later (Colvill and Marshall 1984). Therefore autumn-produced tillers usually have more spikelets per tiller and in addition, they are also found to have more florets per spikelet (Ryle 1964). This is in agreement with a more recent study of Yamada et al. (2004), who found a positive correlation between plant height, tiller size, spike length, and the number of spikelets per spike. These morphological traits have been shown to be highly heritable (Elgersma 1990; Yamada 2004; Byrne 2009). Although the number of

spikelets and florets per inflorescence may vary to some extent from season to season, between species and cultivars, this component is not found to have a large effect on seed yield in perennial ryegrass (Hampton and Hebblethwaite 1983; Elgersma 1990).

3.3 Number of Seeds per Spikelet

The major importance of seed set for grass seed production has led to the definition of floret site utilization (FSU). Elgersma (1991) distinguished between the *biological* FSU, which is the percentage of florets present at anthesis resulting in a viable seed and the *economic* FSU, which is the percentage of florets present at anthesis resulting in a cleaned, pure seed. Different studies reported large variations in biological FSU, but on average 20–50% of the florets are biologically unproductive with losses occurring during pollination, fertilization, and seed development.

In spaced plants, successful fertilization did not decline from the basal to the distal floret within a spikelet (Elgersma and Sniezko 1988) and unproductive florets were found at all floret positions. In later stages of seed development in perennial ryegrass, abortion of 50% of ovules was random and possibly the result of mutational load associated with outcrossing (Marshall and Ludlam 1989).

In contrast, Anslow (1963) found under field conditions a decline in the capacity of florets to set seed from the basal to the distal florets within the spike, and even more pronounced within the spikelet. This decline may be associated with assimilate reallocations via the stems to the inflorescence in the period of anthesis. Florets closer to nutritional resources are favored to develop a saleable seed (Burbridge et al. 1978).

Climatic conditions have a pronounced effect on fertilization with decreasing pollen tube growth at low temperatures, and precipitation disrupting flowering and extending the flowering period. Hence adverse flowering conditions lead to heterogeneous seed development.

Whether genetical, cytological, physiological, or environmental factors are accounted for the low FSU is still not understood. Biological FSU ranges from 50 to 80% in summary over a variety of studies on single plants or drilled plots and glasshouse or open field conditions. Information on the economic FSU is much more limited but seems to be in a range of 20–50% implying a very poor realization of the seed yield potential.

3.4 Seed Weight

Seed development depends on the position of the seed within the inflorescence. Seed weight decreased from the basal to the distal spikelets and with an even steeper gradient within the spikelet (Anslow 1964). The individual seed weight varied with tiller emergence with the earlier tillers having the heaviest seeds, only to a small

degree on the location of the spikelet but to a considerable extent by the position within the spikelet.

From a glasshouse experiment with spaced plants of perennial ryegrass, Warringa et al. (1998) concluded that the amount of carbon assimilates in flowering tillers does not limit seed growth. Reducing light intensity by 75% had only minor effects on seed dry weight. Differences in seed dry weight within the inflorescence of perennial ryegrass under optimal growth conditions (i.e., adequate light and nutrients) mainly arose from differences in growth rate and less so from differences in the duration of seed growth.

A positive effect of the flag leaf size on seed yield in meadow fescue was demonstrated (Fang et al. 2004). Other studies in perennial ryegrass point to the importance of the rachis, spikelets, and glumes as photosynthetic sources which may have more influence on seed yield than the flag leaf tissue, since they are often exposed to a much higher photosynthetically active radiation (PAR) when compared to the flag leaf (Warringa et al. 1998). Assimilates do not seem to limit seed yield in perennial ryegrass, since large amounts of water-soluble carbohydrates accumulate in the basal internodes during seed fill (Trethewey and Rolston 2009).

3.5 Seed Retention

Seed shedding in grass seed crops is a crucial factor in realizing the potential seed yield. Seed shedding occurs due to the fact that seeds within the top of the spikelet mature earlier than the basal seeds, which implies that primarily small distal seeds are lost. However, if harvest for some reason cannot be carried out at crop maturity, also saleable seeds are lost and yield may be decreased dramatically.

Seed shattering occurs due to breakage of the rachilla just below the floret and/or of the rachis just below the glume. In temperate grass species, an abscission layer forms in the rachilla immediately above the glumes and where spikelets contain more than one caryopsis, it forms between the individual florets. At the site of separation, a distinct zone of small cells arises across the intended break, vascular connections are plugged with tyloses and cell wall breakdown occurs as a result of rapid increases in cellulose and polygalacturonase activity. The process appears to be subject to hormonal control in that high levels of auxin inhibit while abscisic acid (ABA) and ethylene promote abscission.

The retention of seed in an inflorescence as a result of strengthening the rachis or rachilla and by the elimination of an abscission layer appears to be controlled by one or relatively few genes (McWilliam 1980).

4 Agronomical Possibilities to Improve Seed Yields

Seed yield improvements can be obtained by optimizing agronomical practices including establishment techniques, nitrogen application, the use of plant growth

regulators, harvesting, and seed cleaning procedures. However, increasing environmental concerns have imposed regulation on the application of fertilizer, plant growth regulators, and pesticides in some seed-producing areas. Crop management is an important factor determining seed yield both in terms of quantity and quality, i.e., purity and germination ability.

4.1 Establishment and Growth

Temperate grasses with vernalization requirements are established during the growing season prior to the seed production year. Different establishment techniques have been developed according to the cropping system ranging from establishment in a pure stand either in spring or in autumn to establishment in a cover crop. To allow for a satisfactory development of slow-establishing undersown grasses, the cover crop may be sown at a wide row distance (24 cm) or at a low seeding rate.

In most temperate grasses, inflorescence production depends on vegetative growth, i.e., number and size of tillers before winter, and therefore nitrogen is often applied to seed crops in the autumn of the year prior to that of seed production (Nordestgaard 1986). For example, application of nitrogen in the autumn to the slow-establishing grasses Kentucky bluegrass (*P. pratensis* L.) and red fescue (*F. rubra* L.) will increase the number of reproductive tillers and hence seed yield (Boelt 1997).

Application of nitrogen in early spring stimulates the development of reproductive tillers, but excess nitrogen can lead to severe lodging, reduced seed set, and increased secondary tillering. When nitrogen application was postponed until 30% or more of the inflorescences had emerged, yields were lower compared with application at the double ridge stage. This yield decrease was correlated with a decrease in reproductive tiller number, fewer spikelets per reproductive tiller, and fewer seeds per spikelet (Hebblethwaite and Ivins 1978).

The nitrogen application strategy, i.e., rate and distribution between autumn and spring, is a very important management tool to stimulate seed crop development. However, recent environmental concern has brought restrictions to the amount of nitrogen that farmers are allowed to use in some seed production areas. Therefore, defining economically optimum nitrogen application rates has become increasingly important (Gislum and Boelt 2009).

4.2 Plant Growth Regulation

Lodging before or during flowering is generally thought to restrict pollination, to reduce the rate of fertilization, and to decrease seed filling. Since the mid-1980s, different chemical components for plant growth regulation have been tested and yield increases have been reported (Hampton and Hebblethwaite 1985; Young et al. 1996). The effect of the growth regulator Paclobutrazol (PP333) on seed yield and yield components was thoroughly investigated and the seed yield increase in

perennial ryegrass was ascribed to an increase in seed number per unit area, usually resulting from an increase in the number of seeds per spikelet (Hampton and Hebblethwaite 1985). However, Paclobutrazol was found persistent in soil and it never became widely used.

From the late 1990s, the chemical compound trinexapac-ethyl, which reduces the level of biologically active gibberellins (GA) through inhibition of the later steps in GA biosynthesis, has been evaluated in a number of grass species. Rolston et al. (2007) reported yield increases in perennial ryegrass by New Zealand seed growers from 2000 kg ha^{-1} to occasionally 3000 kg ha^{-1} when correctly managing input of trinexapac-ethyl, fungicides to maintain green leaf area in the upper canopy, and nitrogen input. Young et al. (2007) observed an average seed yield increase of 459 kg ha^{-1} in perennial ryegrass and of similar range in red fescue. There was no interaction between increasing nitrogen supply and the use of plant growth regulator.

Although remarkable seed yield increases have recently been obtained in a number of grass seed species using plant growth regulators, the effect on yield components and the utilization of the seed yield potential is not yet clear.

4.3 Seed Harvest, Drying, and Cleaning

Seed loss in the harvesting process may be substantial and in some cases, the harvested seed may even fail to meet certification standards in terms of physical purity and germination ability. The variation of seed size and maturity level both within and among the inflorescences is considered a key factor determining seed loss before and during harvest, as well as in the post-harvest management processes of drying and cleaning. During seed cleaning, the saleable seed is separated from impurities, weed seeds, empty seeds, etc. on the basis of seed size, shape, and gravity.

In grass species with poor seed retention, major losses may occur if the crop does not lodge at maturity to prevent seed shattering from wind or rain. Such crops are swathed and left for windrowing for 1–2 weeks before combining. Grass seed species with good seed retention or crops lodged at maturity such as shown in Figure 2 are combined directly at a moisture level of 20–30%. Immediately after harvest seed must be cooled and dried to a moisture content of 11–13%.

Variation in seed maturity level affects optimal drying conditions and may lead to variation in germination ability. Variation in seed size leads to losses in the separation process where physical impurities and weed seeds are separated from the saleable seed. Therefore, a seed crop more uniform in seed maturity level and seed size will realize a higher proportion of the potential seed yield and in addition it often has a higher seed quality.

Since harvest and partly also post-harvest management are on-farm processes that are difficult to reproduce in a small-scale experimental setup, data on the actual loss of potential saleable seed are limited. Still, calculating seed yield from experimental data on yield components often shows a tremendous discrepancy to the harvested yield, and further on-farm demonstrations of harvest operations indicate

Fig. 2 Harvest of perennial ryegrass seed by combining directly (Photo S. Oddershede)

that a substantial part of the potential seed yield is lost in these last steps of the seed production.

5 Opportunities for Breeding

Breeding for high seed yield can focus on the seed yield potential (i.e., the size of the reproductive system), on the utilization of the seed yield potential (i.e., the extent to which each floret develops a viable seed), or on the realization of the seed yield potential (i.e., to which extent each floret develops into a saleable seed).

The seed yield potential and its efficient utilization and realization includes traits, which to a varying degree are under genetic, physiological, or agronomic control. For example, traits such as vernalization response and the number of spikelets/florets per inflorescence are to a high degree genetically controlled and may thus allow the selection of high performing genotypes based on spaced plants. In contrast, a trait with a low heritability such as reproductive tiller number is strongly affected by the management practices as well as the environment and is thus a more difficult breeding target. However, agronomic production systems provide solutions to ensure that the number of reproductive tillers are not limiting seed yield.

Although of major relevance for a high seed yield, seed set and seed weight may not yet be relevant breeding targets, since currently information on the extent of genetic control of those traits is very limited. However, improvement of the following breeding targets has the immediate potential to improve seed yield in forage and turf grasses.

Phenotypic data based on spaced plants are generally found of limited value in predicting field performance and direct selection in drills of progenies in later stages of the breeding program remains the best method (Bugge 1987; Elgersma et al. 1994); however, Marshall and Wilkins (2003) showed that a positive effect on seed yield of two cycles of phenotypic selection on individual plants grown in glasshouse was later confirmed in field plots.

5.1 Breeding for Short Genotypes

The tremendous seed yield increase reported in a number of grass species by the use of chemical plant growth regulators is among the most prominent advances in grass seed production and may provide attractive opportunities for breeding for short reproductive tillers. This aspect is expected to increase in importance, as in most seed-producing regions, environmental concern encourages or even forces the farmer to reduce chemical input in the crop production system. Even though in some species and/or cropping systems the effects of growth regulators are not persistent and the physiology behind needs to be clarified, breeding for genotypes with shorter reproductive tillers may mimic the positive effect of plant growth regulators on seed yield. Obviously, part of this effect is that short reproductive tillers are less exposed to lodging, thereby increasing pollination and thus biological FSU.

In winter wheat, modern cultivars are considerably shorter than older cultivars (40–60 cm) and lodging is not a general problem in the production of cereals except for rye. Forty percent yield increase has been reported when comparing cultivars introduced in 1978 to an older cultivar from 1908 (Austin et al. 1980). In this experiment, all crops were supported by netting and hence yield differences could not be ascribed to variation in lodging. The yield increase was associated with a higher harvest index (ratio of grain yield to grain + straw yield). Cultivars with the dwarfing gene *Rht2* had more grains per ear due to a higher number of grains per spikelet and it is suggested that the reduced competition between the stem and the ear for the limited supply of assimilates leads to a higher grain yield.

For grasses, no dwarfing genes have been identified so far. Furthermore, it still has to be investigated to which degree breeding for short reproductive tillers would affect leaf dimensions and lignification, thereby decreasing forage yield and quality. Moreover, shorter crops standing upright during maturity would be more exposed to seed loss by shedding.

5.2 Breeding for Seed Retention

In cocksfoot, Falcinelli et al. (1994) identified two cultivars with contrasting seed retention and by backcross and phenotypic recurrent selection experimental populations with improved seed retention were obtained. Both breeding methods were effective in significantly improving seed retention compared to the cultivar with the lowest seed retention. There are several reports on variation in the seed retention among plants/cultivars within the same species and hence breeding for at higher seed retention seems an obvious objective.

Breeding for higher seed retention is highly relevant and is further accentuated by the interest in applying plant growth regulators. Seed maturation in any inflorescence may occur over a period of 2 weeks or longer due to the variation in flowering time between plants in the crop, between inflorescences on individual plants, and within individual inflorescences. High seed retention allows harvest time to be postponed until all potentially saleable seeds are ripe. Plant growth regulators effectively

control lodging during pollination, however, high application rates combined with drought may leave the seed crop standing and exposed to wind.

In cereals, domestication has largely eliminated seed shattering, but until now, forage and turf grasses still lack a sufficient ability of seed retention.

5.3 Breeding for Homogeneity

Breeding for homogeneity is considered a key point in sustainable breeding for high seed yield. Homogeneity is important in each production level (e.g., establishment of the seed yield potential, its utilization, and realization). In particular, homogeneous initiation of flowering is essential for effective pollination in outcrossing species. Variation in flowering time imposes uneven ripening, which may explain the low realization of the potential seed yield caused by seed shattering in the field and loss of light seeds during harvest and cleaning.

Genotypes with a higher ovule dry weight and a smaller gradient in ovule dry weight within the spikelet would be beneficial for increasing seed yield (Warringa et al. 1998). This would result in a more homogeneous seed weight distribution at final harvest, where the realization of the seed yield potential could be affected positively. Hence, strong selection for even ripening should be given high priority by grass breeders.

In conclusion, seed yield is a complex trait and affected by many genetical, physiological, and agronomical aspects, some of them not even mentioned in this chapter (e.g., resistance against fungal diseases, eliminating dormancy). Generally, sustainable breeding for higher seed yield should target efficient utilization and realization of the seed yield potential rather than an increase in size of the reproductive system which may compromise forage and turf quality.

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Control of Cultivar Release and Distribution

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1 Purpose of Cultivar Regulation

The legal controls on the release and distribution of plant cultivars are arguably greater than those imposed on most other commercial products. There is specific plant-related regulations imbedded in the laws of many countries worldwide and certainly in all those in the now-developed world with active plant breeding sectors. These regulations control the right of ownership, quantify fitness for use and supervise distribution to the end user. Procedures and standards are set for each stage in the process, many of which conform to internationally agreed guidelines. Compliance with the regulations is policed by officials with powers to act against transgressions.

Most other commercial products from pencil sharpeners and washing machines to cars and farm machinery are only subject to generic laws such as health and safety, prior to release. To gain legal protection for a new product, owners must satisfy stringent patent regulations if sufficiently novel or utilise copyright or trademark declarations, but thereafter defend against infringement through self-financed legal action. A point in case was the October 2000 UK High Court action by Dyson Appliances against Hoover Europe for infringement of its patent for the 'cyclonic technology' in its bagless vacuum cleaner. It took 2 years of expensive court battles before Hoover paid Dyson £4m damages. If the dispute had been over a new grass cultivar, novelty would have been established through the plant control schemes before commercial release. The question arises why plant cultivars are given such specific legal protection.

1.1 Need for Regulatory Control

The overriding driver for the initial legal intervention was food security. Prior to and directly after the two world wars food security simply meant a 'reliable and

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adequate supply'. Supporting a sustainable and innovative plant breeding industry to produce locally adapted higher performing cultivars was seen as one means to this end. This depended on breeding being profitable and able to recoup the costs for developing new cultivars. When released, growers of new cultivars would earn income from the ensuing product, be that flour to the mill, milk from grass to the dairy or a new pitch to a sports club. Without a reliable and equitable income stream, through all these stakeholders back to breeders, there could be no investment in the next breeding cycle. The need for a specific legal framework was apparent when voluntary levy schemes failed to achieve this in the 1940s. Unlike most commercial products, once sold cultivars can be multiplied and resold (except for, e.g. F1 hybrids) and so most of the financial rewards of a new improved cultivar circulated among the growers with only a fraction filtering back to breeders (Figure 1). Even if the breeders controlled the initial seed multiplication step, once released to growers, they had lost control of their seed. This problem may have been somewhat less for grasses and legumes as grassland farmers were less adept at seed production whereas every arable farmer was effectively a seed producer. Overall this resulted in an underperforming, ailing breeding sector.

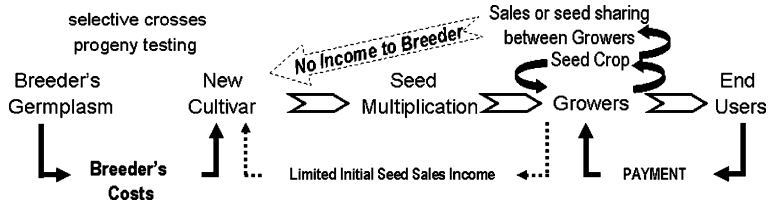

Fig. 1 Operation of unregulated seed trade or voluntary levies

A current parallel would be the music industry for which copyright protection has failed to combat piracy. The CD medium is so easily copied by anyone that the profitability of producing entire albums is now questioned. Single song downloads are predicted to replace CD albums and some artists have given their music away for free in the hope of earning their income from live performances. While a crisis in music provision has undesirable financial implications, a similar crisis in food production is life threatening.

1.2 Introduction of Statutory Controls

Prior to the 1940s agricultural production operated within a wider framework of nationally imposed tariffs and other protectionist measures to free trade (Perren 1995). Plant breeding was largely restricted to the major crops, as voluntary levy schemes (e.g. UK 1940s) failed to support a profitable and sustainable breeding industry. In the early decades of the 20th century, a patchwork of national plant protection began appearing in European countries (Czechoslovakia 1921, France 1922, Austria 1938, Netherlands 1941, Federal Republic of Germany 1953), but these

often only protected the cultivar name and provided little impediment against others releasing identical cultivars under different names (Laclaviere 1965).

The problem faced by plant breeders and the need for food security became largely a hostage to other international trading issues. From the late 1940s a series of multilateral trade negotiations (MTNs) began dismantling national trade barriers and tariffs of around 40% to around 5% by the late 1980s (Laird and Yeats 1990). In parallel to the General Agreement on Tariffs and Trade (GATT) and the Trade Related Aspects of Intellectual Property Rights (TRIPS) agreements came international guidelines on the protection of cultivars (UPOV 1961, www.upov.int). This set the bedrock for the development of the three separate pillars of cultivar control, namely cultivar novelty, cultivar potential and seed quality/authenticity. The ensuing industry structure, represented simplistically in Figure 2, ensured the genetic and seed quality of cultivars.

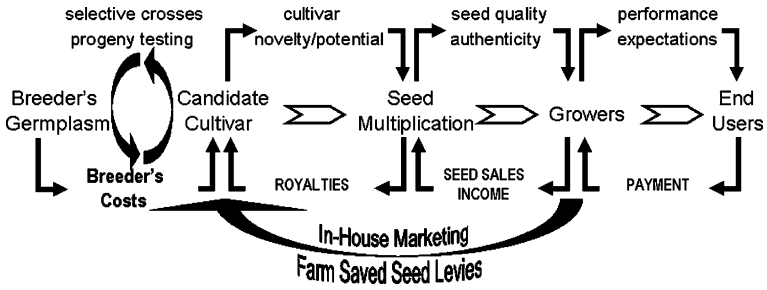

Fig. 2 Testing and certification controls on cultivar release

An overriding control was now in place that prohibited ownership of a cultivar until it was officially proven to be novel. Testing crop species, including forage grasses and legumes, for fitness for use, gave independent confirmation of which cultivars where superior and excluded those that were not. The seed testing function ensured that seed lots of the new cultivars were produced true to type, were of high purity and germination and that every bag contained only the cultivar/s declared on the label. This created consumer confidence and rewarded the best cultivars with greater market volumes and higher prices (Culleton and Cullen 1992, Gilliland et al. 2007). This in turn drove competing breeders to seek even higher capabilities in their new releases, for which they were rewarded with sales success, but which also benefited growers and end users.

An additional benefit for the breeders was that this regulated system facilitated international seed trade and ensured the flow of funds back to the breeders. In later years agreements on levies from farm-saved seed of named varieties were also established. The accumulative effect of all these regulatory controls transformed the plant breeding industry into one with a more secure income stream and eventually led to the rise of large multinational breeding companies.

In grasses and legumes the market value of the seed is much less than for the major arable crops. This is partly due to the perenniality of grasses and legumes

removing the need for annual resowing, but also because herbage is not an end product, unlike grain. The difficulty in demonstrating increased ruminant product profit to offset reseeding costs has made it difficult for herbage seed prices to keep pace with world market prices or to stay instep with the major arable crops. So, as royalty incomes are much lower, forage breeders have had even greater need than arable breeders to optimise returns by expanding into seed multiplication and marketing. Even so profitability remains comparatively low and capturing as large a market as possible has been essential to economic sustainability. Hence many small grass and legume breeding programmes have been consolidated into larger conglomerates that can operate on a wider international market, with a greater economy of scale.

2 The Cultivar Concept

Before considering the processes and implications of the grass and legume control schemes, a clear definition of cultivar is essential. From a botanical standpoint the term is not very auspicious as indicated by Charles Darwin in The Origin of Species. He wrote 'I shall not enter into so much detail on the variability of cultivated plants, as in the case of domesticated animals. The subject is involved in much difficulty. Botanists have generally neglected cultivated varieties, *as beneath their notice*' (Darwin 1859). So it seems reasonable to conclude that the concept of cultivar is driven by pragmatism and serves the practical needs of the plant breeding industry. In botanical taxonomy, 'variety' is a low-level rank below species, which differentiates populations with distinguishing features that merge when interbred. 'Cultivar' was first coined by the American botanist Liberty Hyde Bailey (1858–1954), as a portmanteau of *culti*vated and *var*iety. He defined a cultivar as a cultivated plant that was selected and given a unique name due to its decorative or useful characteristics and was usually distinct from similar plants and retained those features when propagated. He concluded that the term 'variety' is best reserved for botanical taxonomy and 'cultivar' for the products of plant breeding. It is somewhat unfortunate that this convention has not been strictly adhered to as the demarcation between synthetically created and natural occurring has been somewhat blurred. The terms 'cultivar' and 'variety' have become synonyms in commercial use, as evident in the terminology of the official regulatory systems, and this is now irreversible.

Today the naming of a cultivar should conform to the International Code of Nomenclature for Cultivated Plants (ICNCP). The ICNCP defines cultivars in Article 2.1 of its code as, 'an assemblage of plants that has been selected for a particular attribute or combination of attributes, that is clearly distinct (*from all other cultivars*), uniform and stable in its characteristics and that, when propagated by appropriate means, retains those characteristics'.

There is a notable consistency between both cultivar definitions, despite the large time span and advances in plant science that separates them. Both require cultivars

to be distinct from all others and reproducible true to type. These are universally accepted prerequisites, but the common specification that a cultivar has 'a particular attribute' or 'decorative or useful characteristic' is also vital. This is a critical issue, particularly for the registration of outbreeding grasses and legumes and will be revisited later when molecular taxonomy is discussed.

3 Plant Breeder's Rights

3.1 Principles of the UPOV System

Although the USA had a 'Plant Patent' law in 1930 and other European countries had equivalent legislation, none were entirely satisfactory for international harmonisation (Mastenbroek 1988). Consequently a framework for international agreement on plant protection was created by the International Convention for the Protection of New Varieties in 1961. Under this Convention, member states formed the Union Internationale pour la Protection des Obtentions Vegetales (UPOV) (International Union for the Protection of New Varieties of Plants) and agreed to adopt the guidelines into their national legislation. In January 2009, there were 67 UPOV member states (including the EU), the majority of which have joined since 1990 (Figure 3). There are members on all continents but most are from now-developed countries.

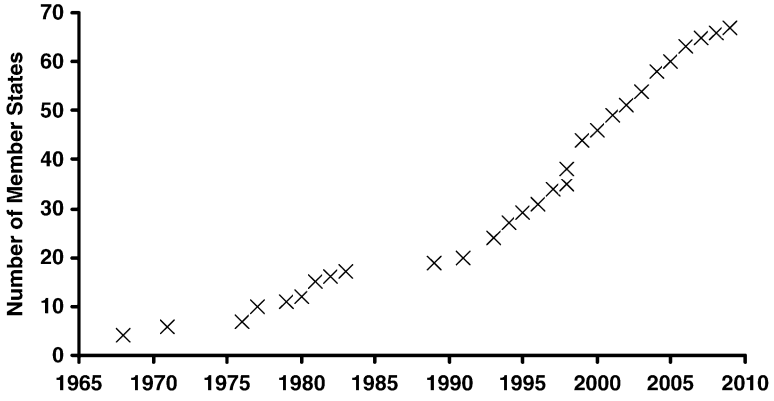

Fig. 3 Rise in UPOV membership since the 1961 guidelines

The Convention was devised to encourage 'the development of new varieties of plants for the benefit of society'. The ensuing Plant Breeder's Rights (PBR) schemes are based mainly on growing tests and generate a description of the relevant characteristics (mostly morphological), which then define the cultivar. To gain PBR, a new cultivar must meet four criteria.

i. The new cultivar must be novel.
   This means that it must not have previously been available for more than 1 year in the country of application or 4 years elsewhere.
ii. The new cultivar must be distinct from any other whose existence is a matter of common knowledge at the time of application. This means that it is clearly distinguishable by UPOV-specified, species-specific characteristics from any other cultivar, whether protected or not. So a new cultivar must still be distinct, even from those no longer protected, if seed sales or farm-saved seed exists. While the breeders of an unprotected cultivar have no controlling rights, this rule prevents them or others 'recycling' it back into PBR protection. It also seeks to promote breeding for novelty and by implication improvement.
iii. The cultivar must display homogeneity in its essential characters, consistent with its sexual or vegetative reproductive mode.
   This means that individual plants of the new cultivar must show no more variation in the UPOV-specific characteristics than normal for registered cultivars in that species
iv. The cultivar must be stable so that the plant remains true to type in its essential characteristics after repeated cycles of propagation.
   This means that future generations of the cultivar in seed production cycles, must continue to display its UPOV traits in the same way as when first examined.

As PBR is only awarded in the country of application, separate applications are required for each country where protection is desired. This may not always involve repeat tests as one UPOV state can adopt a test report from other or have a bilateral agreement for testing services. For example, in the EU white clover is mainly tested by the UK. For forage species and amenity grasses, Belgium, Denmark, France, Germany, Netherlands and UK have various bilateral agreements and test different grass and legume species for each other. As each new candidate must be distinct from all others in 'common knowledge', the issue of 'precedence' arises when candidates are progressing in parallel through the testing scheme, either as applicants to the same country or spread over different UPOV states. In these situations the cultivar with the earliest application date in any of the countries has priority over a later applicant. If the two candidates are indistinguishable then the later applicant is refused PBR.

3.1.1 Limitations to PBR

In the case of grasses and forage legumes rights are granted for a minimum of 20 years (frequently 25 years). There is a specific list of UPOV-protected species (www.upov.int) and not all grass and legume species are currently included. This is expanding annually, however, as UPOV technical experts derive test protocols for species where a diversity of cultivars is being bred.

PBR protects the breeder's financial interests and awards control of all propagation, marketing, importing/exporting and maintaining of propagation stocks.

Nonetheless, PBR is often described as a 'benevolent right' as it does not prevent third-party use of the cultivar for private non-monetary benefit, for experimentation, breeding of new cultivars or subsistence farming (if no cash crop involved). Furthermore, while breeders can impose a licensing fee on anyone wishing to reproduce their cultivars for sale, a 'compulsory license' can be granted to a secondary producer if the national interest requires public access to protected cultivars and the breeder can not meet that demand. There are, however, no reported examples of this in grasses and legumes.

Finally the breeder must name the new cultivar based on ICNCP guidelines which prevent it from being deliberately misleading or too like another cultivar. This becomes the official name that must be used in all marketing or referencing of the cultivar. Re-use of the name is not permitted until the cultivar has been out of common knowledge for 10 years, to avoid any association between the merits of the initial cultivar and any later one given the same name.

3.1.2 PBR Methodology for Grasses and Legumes

To obtain PBR, a breeder submits an application and completes a 'Technical Questionnaire' (TQ) that gives information on which grouping of registered cultivars should form the reference collection for the test. The TQ may also include details of any special features of the cultivar that the breeder might want to examine as a unique distinguishing characteristic. The candidate is then compared with the registered cultivars according to UPOV guidelines that determine distinctness, uniformity and stability (DUS).

3.1.3 Definitive Seed Stocks

Breeders are required to supply candidate seed with good germination and vigour. For large-seeded grasses, e.g. *Lolium*, *Dactylis*, some *Festuca*, the requirement is normally around 1.5–2 kg and for smaller seeded genera, such as *Phleum*, *Trifolium* and *Lotus*, 0.75–1 kg.

This seed becomes the 'definitive stock' and defines the candidates identity throughout its testing period and thereafter until it is no longer protected and also out of 'common knowledge'. As PBR can last for 25 years and 'common knowledge' is open-ended, this seed is normally stored under conditions that preserve germination. Typically it is dried to a moisture content of around 4–5%. Stable and cool temperature control is also important but for additional longevity may be cooled to sub-zero temperatures, typically –20°C.

This seed remains the property of the breeder and if the cultivar is refused PBR it must be returned or destroyed. It cannot be used by anyone for purposes other than PBR and seed certification. If the cultivar gains PBR, this seed enters the reference collection for future DUS trials and is also used to confirm that commercial seed stocks are produced 'true to type'. So, this definitive seed stock defines the cultivar, rather than any paper or computer record, particularly for grasses and legumes, where unique distinguishing features are rare. When the amount of stored seed of the

definitive stock becomes low, a replacement sample is requested from the breeder and this is examined in a DUS trial to validate that it matches the original sample. Only then is it allowed to be used as a replacement. Repeated failure to supply a matching sample will set in place actions leading to PBR withdrawal if the breeder cannot correctly reproduce the cultivar that was originally protected.

3.2 Assessment of UPOV-Approved Characteristics

Each species that UPOV has guidelines for has a defined character set to be measured in order to determine DUS. If the candidate is found to be novel (plus uniform and stable), PBR is awarded. If not, then its refusal protects the rights of existing cultivars. For grasses and legumes virtually all the characters are morphological and these can be broadly grouped into either, measured or visually assessed.

Visually assessed characters include various aspects of growth habit (Figure 4) but can also involve an assessment of a physiological process such as flowering date (Figure 5). Measured characters include for example quantifying leaf, petiole and stolon dimensions on white clover (Figure 6).

The number of characters specified by UPOV varies with species, but typical numbers for grasses and legumes are between 10 and 22, for example *Dactylis glomerata* 10 traits; *Lolium perenne* 21 traits; *Medicago sativa* 22 traits; *Trifolium pratense* 18 traits.

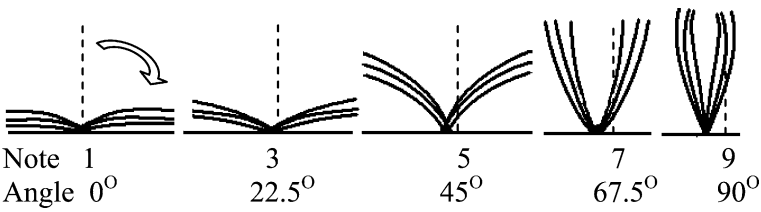

Fig. 4 'Vegetative Growth Habit' in ryegrass cultivars (UPOV No. 2). This assessment involves a visual estimate of the angle that the outer grass shoots make to the vertical, reported as numerical notes

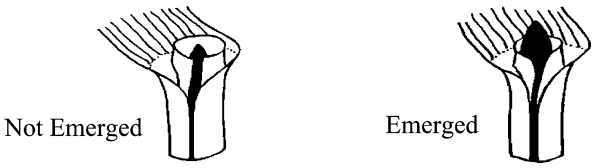

Fig. 5 'Time of Inflorescence Emergence' in ryegrass (UPOV No. 11). For this character the emergence date is recorded on a plant, when three inflorescences have visibly emerged beyond the ligule

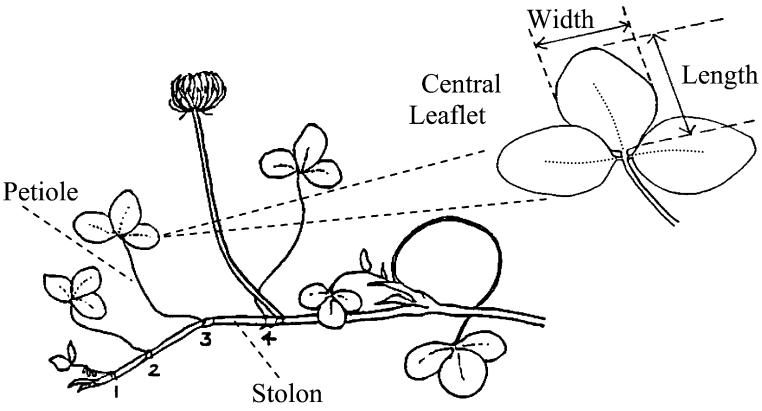

Fig. 6 Morphological characters measured from the third node on a white clover stolon. Thickness of stolon (+/–0.1 mm, UPOV No. 11), length of petiole (+/–1 mm, UPOV No. 12) and thickness of petiole (+/–0.1 mm, UPOV No. 13). Measured once flowered, from the third node with a full expanded leaf

3.2.1 Assessment of Additional Characteristics

The UPOV-approved characteristics for any species are not a restrictive list and national authorities may include additional characteristics if they prove useful and meet UPOV criteria. This means that any new character must be able to determine uniformity and stability in any cultivars that they discriminate. To do otherwise would be contrary to the UPOV principles of DUS.

The list of UPOV characters is also not static and is reviewed and modified on the evidence of experts from member states. Calculating minimum character sets as in Table 1 can provide evidence of the discriminating power of additional characters.

Table 1 White clover minimum character set for pairs separation

UPOV character	Character name	% Pair distinctions
Additional	Proportion of plants with cyanide-glucoside	82.41
11	Thickness of stolon	12.64
6	Time of flowering	2.73
1	Form inflorescences before vernalisation	0.83
Additional	Plant growth habit	0.57
Additional	Inflorescence height above canopy	0.25
2	Intensity of green colour	0.16
8	Plant width	0.12
20	Number of inflorescences	0.11

The remaining UPOV characters uniquely separated less than 0.1% of the 5625 pairwise comparisons

This involves identifying the character that discriminates the greatest number of cultivar pairs in a taxon. These comparisons are then excluded from the analysis which is repeated to identify the second most discriminating character and so on until all pairs that can be distinguished have been separated. For 'additional characters' that prove as powerful or better than existing UPOV characters, are easily measured and meet uniformity and stability requirements, the appropriate Technical Working Party will consider their UPOV recognition.

3.3 Trial Design and Test Procedures

The trial designs depend on the reproductive mode of the cultivar, such as self-pollinated, cross-pollinated or vegetative and whether the characteristics are visually assessed or measured. Generally, this involves growing-out trials using rows or groups of plants mainly for visually assessed characters and single spaced plants (Figure 7) for both measured and visually assessed characters.

Fig. 7 Spaced plant trial for DUS testing of perennial ryegrass. Cultivars are planted in six randomised replications (rows), each with 10 individuals (Photo T. Gilliland)

For species where little variation is expected within cultivars, such as some self-pollinated legumes, distinctness is more easily assessed visually rather than by statistics. Assessments can be made through side-by-side comparisons of groups/plots/rows of example cultivars that clearly express the different possible states in a qualitative characteristic. Alternatively, randomised trials can be used and analysed but are much more laborious. Quantitative characters can be converted into pseudo-qualitative characteristics by sub-dividing them into a number of visual states along a linear scale (e.g. Figure 4). The accuracy of these assessments is highly dependent on the DUS examiner's expertise.

For species where there is considerable variation within cultivars, such as most grasses and many legumes, data are obtained for individual plants in order to determine the mean expression and the variation within the candidate cultivar compared to that of the reference collection.

The magnitude of differences between and within cultivars is also influenced by G × E variation. While qualitative characters, such as presence and absence of awns in *Lolium* spp., are often highly heritable and may be genetically fixed, quantitative and pseudo-qualitative characters are particularly reactive. For this reason, plus the need to reduce chance effects in very large trials, grass and legume DUS tests normally require three growing seasons with new plantings each year. In some cases, however, candidates may be distinct from all controls after two trials. So PBR decisions are made 3 or 4 years after the application, to give time for an establishment year and data analysis (Table 2)

Table 2 Example DUS testing scheme

Year 0	**Test Year 1**	**Test Year 2**	**Test Year 3**	**Year 4**
Sow Trial I	Test Trial I			
	Sow Trial II	Test Trial II		
		Sow Trial III	Test Trial III	
Application			Award/Refuse PBR	

Single cultivar records by visual assessment or side-by-side visual comparisons are generally quicker and cheaper than individual plant assessments. However, this requires that the cultivar has a single state of expression in all plants. As the majority of grasses and legumes are cross-pollinated, measurements and visual records are made on individual or groups of plants with replication to account for within-cultivar variation.

3.4 Determining DUS

3.4.1 Distinctness

If two cultivars are found to be significantly different for any one characteristic then they are considered to be distinct. For truly qualitative characters in which totally different highly heritable states exist and have no intermediate states, distinctness is established simply by the visually recorded difference (e.g. open or closed stems). For pseudo-qualitative characters, where a range is divided into a number of states, distinctness normally requires a difference of at least one intervening state (e.g. state 1 is distinct from state 3, but neither is distinct from state 2, Figure 4). This is because the character is a continuum that has been segmented into a number of states, but with a graduation between them. For the majority of grasses and legumes, due to their cross-pollination, a single state repeated in all plants of a cultivar is not normally expected. Therefore, distinctness is assessed by comparing cultivar

means calculated on the basis of replicated data. The number of plants depends on the species but for most grasses and legumes 60 individual plants, along with row plots for some pseudo-qualitative characteristics, are distributed over three or six replicates in a randomised block configuration.

Statistical proof of distinctness can be achieved by a range of methods including ANOVA, multiple range tests and other non-parametric procedures that comply with UPOV statistical standards. A common standard is to require the candidate to be distinct by a minimum of one character against each registered cultivar by LSD 1% in at least 2 out of 3 years, as long as the same cultivar is the 'greater' in both years.

UPOV has developed a specific DUS statistical method to account for annual variation in PBR trials. Combined over years distinctness (COYD) analysis accumulates differences over years and while still requiring the direction of difference to be annually consistent, makes adjustments when environmental conditions cause a significant change in the size of separation between cultivars in a test year. This has proven to be a more stringent distinctness assessment than the LSD 1% approach and has increased the number of successful candidate distinctions in cross-pollinating grasses and legumes.

3.4.2 Uniformity

Assessment of the variation between plants within a cultivar for each characteristic is the basis for the uniformity test. This variation can have both genetic and environmental components, but uniformity assessment requires the genetic component to be separated from the environmental component.

As a general rule the states of expression of qualitative characteristics are not interchanged by the environment. So for self-pollinated legumes all plants should express the same state of a characteristic. If not then the cultivar is non-uniform and will be refused PBR, subject to certain tolerances for off-types. For quantitative and pseudo-qualitative characteristics, environmental variation will exist and is taken account of by calculating standard deviations for each character based on the control varieties. This approach requires a candidate cultivar to be no more variable than its comparable controls.

Similar to distinctness, UPOV has adopted a specially devised statistical method, called combined over years uniformity (COYU). This takes into account variation between years, adjusting the standards according to the reaction of the control cultivars to the annual growing conditions in the trial. Therefore, this analysis provides an accurate and responsive assessment of uniformity by raising the threshold when environmental pressures cause high inter-plant variation and lowering the tolerance threshold when environment induced variations are low.

3.4.3 Stability

While the concept of stability is that the examined characteristics remain unchanged after repeated reproduction or propagation, it is not essential to assess separate generations. For cross-pollinating grasses and legumes, it is impractical for the DUS

examiner to produce further generations of seed from the definitive stock, as costs and logistics of isolating every candidate to produce pure cleaned seed is prohibitive. The pragmatic solution is to accept that when the uniformity of a candidate is within the tolerance levels of similar control cultivars and no visual evidence of segregation or excess off-types are detected in the trials, then the candidate is stable. During the lifetime of a cultivar, its stability is further assessed when a new reference sample is submitted by the breeder and when commercial seed lots are validated against the definitive stock (as described in Section 4 below).

4 Control of Seed Production and Distribution

Founded in 1924, the International Seed Testing Association (ISTA www.seedtest.org) specifies validation methods to determine if seed is produced fit for purpose (Steiner et al. 2008). The association develops and publishes standard procedures and its rules have been adopted within the seeds regulations of over 70 countries worldwide.

The ISTA rules promote uniformity in seed testing and along with OECD schemes define the quality and procedures to be used in all aspects of seed control, including certification, identity validation, sampling, germination, purity, vigour and disease thresholds plus what information is provided on reports and labels. This ensures that cultivars are reproduced true to their definitive stock, as submitted for DUS and VCU testing. This promotes both grower confidence in purchasing seed and also facilitates international seed trading.

4.1 Procedures for Controlling Seed Quality and Authenticity

Control of seed authenticity and quality depends on documented traceability of seed lots from initial 'breeders seed' through to the final commercial C1 or C2 generation (Figure 8), plus validation against the definitive stock.

Production of pre-basic seed is strictly quality controlled, often involving plants in rows or similar configurations to facilitate intensive roguing of off-types and same-species volunteers. Pre-basic can also be continuously multiplied to produce

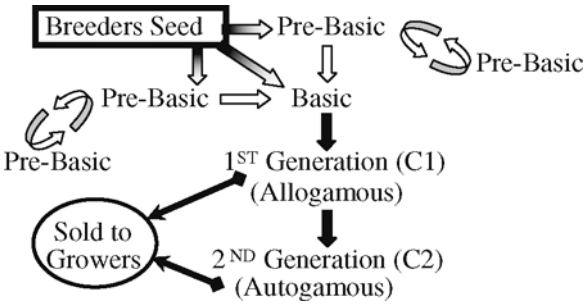

Fig. 8 Control of generations for certified seed production

more pre-basic, but all are subject to the same stringent processes. Basic seed can be produced directly from the breeders seed though normally the intermediate 'pre-basic' generation is undertaken to multiply stocks. This generation undergoes field inspections by officials to confirm isolation and crop purity standards are met, plus the parallel growing of control plots of the same seed by officials, for direct validation against the definitive seed stock. Once larger production areas of basic seed are sown, these can only be used to produce certified commercial seed lots (C1, C2). Field inspectors ensure standards for isolation and freedom from prescribed diseases, weeds and crop contaminants (including other cultivars) have been met. Samples are drawn from each generation and tested in an ISTA licensed laboratory and officials grow samples of C1 and C2 seed in post-control plots to ensure that the seed sold matches the definitive stock.

There are maximum sizes for the C1 and C2 seed lots, depending on the species (10 T for grasses and most legumes). Procedures are provided by ISTA for sub-sampling these lots which are then submitted to the laboratories for further subdivision and subsequent purity and germination testing.

4.2 ISTA Standards for Grasses and Legumes

For most grasses and legumes C2 seed is not permitted, as their allogamous nature makes them more prone to pollen contamination and genetic drift than self-pollinated species. Grass and legume seed lots are limited to 10 T and must achieve a minimum germination between 75 and 85% and purity between 90 and 98% depending on which species and which seed generation is being tested. Unlike many self-pollinating crops, grass and legume cultivars seldom have a clear uniform characteristic that might alert a grower if the wrong cultivar was sold or if seed of another cultivar had become a contaminant. So grower confidence in this testing and certification system is critical to a successful forage seed sector. As seed failures and accidental mislabelling or contamination are extremely rare, the certification label on every bag is universally accepted as an assurance of good seed of the named cultivar.

5 Evaluation of Cultivar Value

Although Plant Breeder's Rights confers ownership of a new cultivar and affords its protection from exploitation in all UPOV territories, it does not also give automatic rights of release, even into the country where the PBR application was made. Permission to grow and sell a cultivar is the reserved right of every sovereign state. Uniquely within the EU, inclusion on a member state's national list, provides entry to the EU Common Catalogue (CC) and marketing rights throughout the EU.

5.1 Independent VCU Testing Systems

Each country can compile its own 'National List' (NL) of cultivars, awarding permission to commercialise. The test procedures involve a DUS test as in PBR, though

do not require the cultivar to be a recent release and can have been commercialised in another country for many years (PBR requires 'novelty' and so does not permit this time lag). Obtaining NL status usually, though not always, involves an evaluation of end use potential, called 'Value for Cultivation and Use' (VCU). Amenity grasses intended for turf production must undergo national performance trials in France, whereas in the UK there is no such requirement. For forage cultivars there is always a VCU test, typically taking 4–5 years to complete, similar to the DUS test. In some countries NL testing is followed by further regional 'Recommended List' (RL) testing of the very highest performers to identify regionally elite cultivars within a country. RL trials can take a further 3 years or more and require periodic retests to reassess performance against the newest releases (Weddell et al. 1997). Gaining RL status is highly prized by breeders and merchants as it has a major impact on seed sales (Culleton and Cullen 1992, Gilliland et al. 2007). In order to shorten the test procedures, RL trials are run in parallel with NL trials in some countries.

The NL application process is also different from PBR in that some countries do not recognise precedence. In these countries, if two applicant cultivars enter NL trials together for the first time and are eventually found not to be mutually distinct, both are refused NL, regardless of the date of the application. In PBR testing, the earliest applicant in any country passes.

So unlike PBR- and the ISTA-coordinated seed control functions, there is no guiding organisation that coordinates and harmonises VCU testing across different countries. This is partly because PBR and seed quality tests have precisely definable end points, whereas 'value for use' varies depending on the climatic conditions, cultural practices and market requirements in any country. So the evaluation test procedures differ not only between species but also between countries or even regionally within countries. Breeders, therefore, need to be aware of the diverse rules and test procedures in different countries and to make (and often fund) multiple applications to market their varieties internationally.

There have been some efforts to harmonise VCU testing of forage grasses across national borders in the EU by making listing decisions on a regional eco-environmental basis, but as yet without success. In contrast, progress has been made on a European Seed Association (ESA, www.euroseeds.org) coordinated amenity grass testing scheme across several EU member states and the National Turfgrass Evaluation Program (NTEP, www.ntep.org) in the USA oversees harmonised guidelines for testing.

5.2 VCU Testing Objectives

A consequence of the multipurpose function of amenity grasses is that VCU testing procedures, such as that specified by NTEP, assess a large number of characteristics. Typically, these include turf colour (including spring green-up, winter colour and seasonal/stress colour retention), cleanness of cut, density, texture, tolerance of environmental stress (heat, cold, drought), resistance to pests and diseases,

competitiveness against monocot and dicot weeds, traffic tolerance and thatch accumulation. A further complexity is that the relative importance of these many characteristics changes depending on the intended use. Clearly the requirements of a roadside verge which is roughly cut a few times a year and that of a cricket square which is finely mowed and then scalped before play require cultivars with different attributes. The same is true for the differing demands of parklands, sports pitches, tennis lawns or golf fairways, tees and greens.

Grasses and forage legumes are among the most difficult species to provide a definitive assessment of value. For crops such as bread wheat, the grain yield and quality requirements from the single harvest are precisely defined by the end user. For grasses and legumes, there is a multiplicity of grower requirements from what is considered a multipurpose crop. Most grasses and legumes have multiple harvests and are expected to be self-regenerating with varying perenniality. For those intended for forage production, there is the added complexity of being fed to ruminants before the end product is achieved. VCU testing of forage grasses and legumes must, therefore, account for the very diverse end-user requirements. Variables include whether the herbage will be conserved or grazed and by what type of ruminant, how intensely or extensively will be the management practice, what biotic and abiotic stresses must be tolerated and how long the pasture must last. Typically total dry matter yield and persistence are universally important attributes of forage grasses and legumes. In addition, cultivars are assessed for environmental stress tolerance (heat, cold, drought), pest and disease resistance, weed competitiveness, and nutritive value characteristics. The latter category primarily involves an assessment of digestibility, though other nutritional parameters are gradually entering test procedures, if not routinely then as special tests on specific cultivars.

5.3 VCU Testing Procedures

In order to build sufficient data to make a valid assessment of cultivar performance over a range of typical growing conditions, testing procedures normally involve several sowings followed by several harvest or mowing years at several locations.

Amenity grass trials can utilise small (e.g. 2 m square) plots to reproduce the operational conditions consistent with final use. As many of the characters assessed are highly heritable, visually scored features, the operational costs of these trials are probably less per cultivar than for forage species. While differences in biotic and abiotic stresses will change with location and affect turf quality, many of the visual characters vary less with location than agronomic attributes. This greater consistency of performance and more definable end uses can facilitate the development and adoption of geographically wide standardised testing such as those of the ESA and NTEP.

For forage grasses and legumes, VCU testing (Figure 9) is normally done using a plot harvester to assess conservation production and simulate grazing. The relevance of cultivar rankings under simulated grazing compared to actual grazing has been questioned, particularly for legumes (Wilkins and Humphreys 2004). Direct

Fig. 9 VCU testing of grasses in Northern Ireland. Management of trials includes conservation production, simulated grazing, i.e. frequent mowing as well as actual grazing (Photo T. Gilliland)

assessment of ruminant output for each cultivar would, however, incur excessive expense and resources and so cannot be used to routinely screen large candidate numbers. Grazing has been used to compare the smaller numbers of highest performing cultivars in recommended list trials, though the animals are usually only used as a treatment, with herbage performance rather than animal performance being measured (Weddell et al. 1997).

The small progressive increases in performance, typically at an average of approx 0.3–0.5% per year for DM yield, make testing precision vital to identifying improved forage grass and legume cultivars (Aldrich 1987, Talbot 1984). VCU trials, therefore, normally involve randomised plot trials. Sowing strips of cultivars in fields on-farm can also be an effective in situ assessment method, but to gain adequate precision requires an accumulation of many repeated tests, ideally in different locations (Weikai et al. 2002). Furthermore yield-related characteristics are very responsive to management and biotic or abiotic stresses, and so cultivar performances differ greatly from region to region and year to year. Research by Talbot (1984) concluded that the variance associated with harvest year is greater than that for sites, which is greater than for replicates in forage grass trials. Talbot also showed that infrequently cut conservation tests had higher variances than multi-cut-simulated grazing. In practice this meant that a perennial ryegrass with a true yield of 105% of the standard had a 4% chance of randomly failing to meet the '100%' pass standard if six test sites were used but a 10% chance if only three test sites are involved. Ideally, VCU trials are carried out at a minimum of six test sites, each with three or four replications, and involving 2–3 harvest years.

Pass/fail decision methods differ between systems. This can involve assessment relative to an accepted set of standard cultivars, to a statistically calculated standard, or for differing recommended list systems, from 'better than the best' to 'better than the worst'. Where statistical standards are set, such as greater than LSD 10% of a defined threshold, reduced trial precision increases the magnitude

of this confidence limit. This raises the pass threshold. So reduced precision results in increased breeder's risk of failure, rather than increased tester's risk of recommending a below-standard cultivar. Given that precision depends on optimising the number of controls, sites, replicates, sowings and trial years, plus the need for multiple DM yield assessments and nutritive value analyses, forage cultivar trials are expensive procedures.

6 Challenges for Cultivar Testing and Control

6.1 Management of PBR Reference Collections

According to UPOV, a candidate cultivar must be compared to all those in common knowledge. As most UPOV characters are morphological with high G × E interactions, field-based DUS trials must make direct comparisons between all reference cultivars and candidates in the same year and site. This is a gargantuan task for DUS testing of grass and legume species, as evident from OECD lists (e.g. Table 3).

Table 3 Example cultivar numbers January 2009

Species	OECD	EU		OECD	EU
Lolium perenne	1311	965	*Medicago sativa*	874	380
Festuca rubra	387	333	*Trifolium pratense*	265	202
F. arundincacea	371	195	*Trifolium repens*	208	134
Poa pratensis	294	191	*Vicia faba*	195	152
Dactylis glomerata	209	130	*Vicia pannonica*	145	3
Agrostis stolonifera	66	44	*Lotus corniculatus*	91	38

It can be assumed that cultivars developed for geographically very different regions will be distinct, e.g. the EU CC can be taken as 'common knowledge' by EU countries, so excluding cultivars grown in other continents. Even this pragmatic approach does not reduce numbers to manageable levels in all species (Table 3). This is a major problem for most grass and legume PBR schemes, as cultivars tend to have very long commercial lives and so reference collections increase year on year. Devising strategies to curtail the size of the collection is a major challenge.

In species where qualitative characters exist in a number of precisely defined states, these can be used as grouping characters. For example, different leaf shapes, such as cordate, elliptical, lanceolate, obovate and ovate, provide excellent grouping characters to limit DUS comparisons to only those reference cultivars of matching leaf type to the candidate. Unfortunately, these are characteristics of tree species and there are few sub-dividing qualitative characters in grasses or most legumes. An alternative approach has been successfully developed (Camlin et al. 2001). This uses a cyclic planting scheme, to reduce the annual planting of the reference collection by a third (Table 4). It involves calculating data for each unsown group of control

Table 4 Cyclic planting scheme for managing reference collections

Control cultivar groups				Candidate test years		
Year	2004	2005	2006	2007	2008	2009
Group A	Past	Past		Test	Test	×
Group B		Past	Past	×	Test	Test
Group C	Past		Past	Test	×	Test

Past = previous trials on controls, Test = controls + candidates
× = unsown group in that year, replaced by 'Past' data analysis

cultivars by assessing the inter-group relationships in the two preceding sowings of that group.

Management of reference collections is currently one of the biggest problems facing the testing authorities. It is also a major testfee expense for breeders submitting into different countries. While statistical advances in managing reference collections may reduce unfavourable control-candidate ratios, excessive workloads are likely to remain a considerable impediment to efficient DUS testing for grass and legume species. Bilateral agreements already exist between UPOV member states, as reported previously, and this may have to become common practice.

6.2 Adoption of Plant Biotechnologies

The creation of GM cultivars has been a major advance in breeding technology. The controversy surrounding their release in many countries is well known, and the wind and insect pollinating nature of many grasses and legumes gives them a high risk of adventitious contamination. There are already reports of transgenic grasses such as herbicide-resistant creeping bentgrass for the amenity market (Reichman et al. 2006) and GM improved ryegrasses for the agricultural market (Spangenberg 2005). Although the regulations controlling release and patent rights will prove complex and time consuming, the issues for PBR are less arduous. UPOV has taken the view that existing DUS procedures are adequate to determine the novelty of the ensuing GM cultivar phenotype, recognising no greater rights than currently for conventional cultivars. The complex issues regarding permission to release and patent controls will remain the responsibility of national authorities.

A more difficult issue has been the use of electrophoretic techniques to determine the novelty of candidate grass and legume cultivars. While both molecular and biochemical methods have proven highly discriminating, concerns have long existed regarding a lack of protection from plagiarism (Wright et al. 1983). For self-pollinating crops, UPOV has adopted some specific tests as the inherent uniformity within such cultivars protects against exploitation. In contrast, individual plants in allogamous grasses and legumes can be separated into a number of distinct types for a single gene locus. It is therefore possible to multiply a subset of anyone of these types to faithfully reproduce the phenotype of the original cultivar, but with

a changed electrophoretic identity (Quaite and Camlin 1986). It can be argued that EDV guidelines may help offset this risk; however, the underlying concept that cultivars should be entities with varying end use features (see Section 2) is not addressed by these guidelines. So these taxonomic techniques could be very detrimental to forage breeders who already struggle to get customers to associate specific benefits to individual cultivars. Electrophoretic methods have, however, been used to confirm the award of PBR to grass cultivars, e.g. in France by using isozymes. This has only been when multiple genotypic marker differences have supported evidence of several morphological differences at just below DUS pass thresholds. In this case the risk of plagiarism or erosion of cultivars as distinct entities is considered negligible.

6.2.1 Assessing Essential Derivation

The concept, of Essential Derivation was introduced into UPOV 1991, to further protect registered cultivars from plagiaristic exploitation. It is fundamentally different from PBR as it is the responsibility of the breeders to enforce it, rather than the testing authorities. It operates when a new cultivar is found to be distinct from a protected 'initial variety' (IV), but retains the essential characteristics of the IV. If the breeder of the IV can also show evidence of genetic conformity and evidence of derivation, then the new cultivar can be declared as an 'essentially derived variety' (EDV). The IV breeder will then exercise the Intellectual Property Rights (IPR) of the IV over the EDV, negotiating its withdrawal or a royalty sharing agreement. This is not in conflict with 'breeder's exemption' as it still allows breeders to use registered cultivars that they do not own in their crosses. It does, however, protect against plagiarism.

Grasses and legumes are some of the most difficult species to identify EDV dependencies, due to their allogamous nature and lack of clearly distinguishing features. For this reason the ESA funded a molecular AFLP method in 2000 to identify putative EDV relationships in perennial ryegrass (Roldán-Ruiz et al. 2000) and the ISF is currently investigating an improved SSR-based test for worldwide use.

6.3 Improving Forage VCU Assessments

Due to the high priority given by most governments to environmental policy issues, official VCU systems are increasingly expected to contribute to reducing the environmental impact of food production. For forage grasses and legumes this is associated with reducing nutrient losses from grassland through improved fertilizer efficiency and enhanced digestion of the forage to give lowered nutrient excretion. In addition advances such as in genomics and metabolomics and the development of, e.g. NIRS rapid analytical systems are gradually providing new breeding innovations that must be evaluated.

This need for more information on the impact of improved grass and legume cultivars on ruminant performance does not necessarily mean that additional

batteries of new performance tests are desirable. Parameters such as total digestibility, crude protein, soluble carbohydrate and fibre contents, plus alkaloid toxin levels and fatty acid contents are useful nutrient factors for predicting ruminant performance (Williamset et al. 2001). With NIRS technology and the added possibility for in-field measurement on plot harvesters, assessing all these characteristics is becoming financially and logistically more feasible for the testing authorities. Every additional parameter, however, brings another compromise to the selection process during breeding. To select for many characters usually means accepting less than the optimum performance in each character to find the best compromise cultivar overall. Testing authorities must place a justified value on each characteristic they require and weight the importance of characters according to their relative value of cultivation and use. If all the independent VCU testing organisations impose differing methodologies, management schemes and weightings to all these parameters and frequently change and expand their demands then this could become more of an impediment than a catalyst to breeding advances. For significant progress to be made coordinated VCU standards need to be agreed internationally as for PBR and seed control.

6.4 Food Security Issues

The modern definition of food security incorporates both adequate supply and ability to pay. Undoubtedly the trend of more countries joining UPOV and adopting ISTA quality standards will continue (Figure 3). So cultivar release and distribution control is expected to expand further into the developing world. This brings concerns that fragile rural economies will be competitively disadvantaged by an inability to afford breeders' IPR. This is not an issue for amenity grass breeding and it is also unlikely that forage breeding will be at the forefront of these concerns. In the developing world, pasture farming is more dependent on low-input low-output systems such as range farming and extensive systems. In these systems, production per unit area or per animal is low, but they can often match the profitability of reseeded intensive grassland enterprises. Furthermore, unlike many of the major food crops, IPR has not lead to inflating seed prices for agricultural grasses and legumes as breeders have had to account for the low profitability of the agricultural sector they supply.

7 Impact of Regulation and Control on Breeding Progress

Judging the extent to which regulation and control of grass and legume breeding has been beneficial depends on what comparisons are made. The number of cultivars being produced and protected has certainly risen from single figures in the 1960s to an OECD list in 2009 of 3857 cultivars from 18 grass species and over 2000 forage legumes across 14 species. This has been a continual rise even in more recent years,

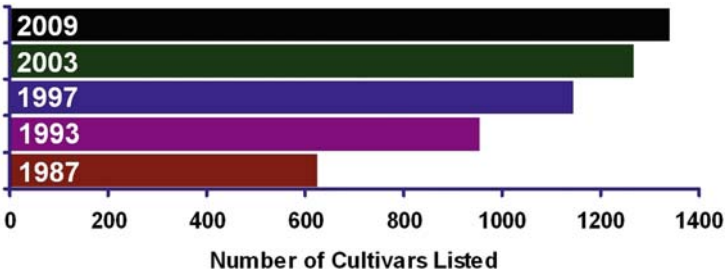

Fig. 10 Grass and clover cultivars on EU Common Catalogue

as evident from the EU CC, though the rate of increase has slowed in some species (Figure 10).

Clearly the described procedures are facilitating the release of large numbers of commercially attractive cultivars and are functioning as intended. However, market prices and volume are primary factors determining the success of new cultivars. Clearly to justify current cultivar numbers requires a substantial market volume. The ISF reports that annual world seed production is around 650,000 T for amenity and agricultural grasses and 130,000 T for forage legumes. So although this evidence shows that there is confidence in the cultivar registration systems, cultivar numbers alone are not a true measure of the overall impact of the plant control systems in grasses and legumes. Regulatory systems should also promote cultivars with improved end-user value and assure confidence in the consistency of the traded product.

There is irrefutable evidence that the seed testing and certification functions achieve their stated purpose as reseeding failure due to substandard seed is very rare and accidental mislabelling of seed equally uncommon. This has generated and sustained a vibrant international trade based on confidence in the authenticity and vigour of certified seed.

There is clear evidence that performance standards on agricultural evaluation lists have risen steadily from the 1960s to current times (Aldrich 1987, Van Wijk and Reheul 1991), with an average rise in ryegrass agricultural yields of between 0.5 and 0.6% per annum. Although this could be construed as a breeding achievement alone, evidence from seed sales shows that the highest performing grass and legume cultivars command greatest market shares (Culleton and Cullen 1992, Gilliland et al. 2007), with new elite cultivars quickly taking the sales of former market leaders. Here again the control system is working as intended as growers are being guided to the independently confirmed highest performing material and breeding progress is awarded accordingly.

It is less easy to quantify testing-dependent progress in the more aesthetic characteristics of amenity grasses. Due to the diversity of end use, cultivar lists for amenity grass species either become more descriptive than precisely prescriptive or involve separate end-use-specific lists. Nonetheless, these lists are evidence of both breeder's success in producing improved cultivars for specific functions and

the achievements of the testing organisations in gaining market penetration for the best examples.

A major difference between the amenity and agricultural sectors has been that the former serves an expanding market that has to date been relatively well financed. In contrast, the over-production of agricultural products since the early 1970s, the introduction of quota in some sectors, and the progressive dismantling of trade barriers have seen agricultural commodity prices fall particularly for pasture-based enterprises. Successful farming has increasingly depended on efficiency of production by minimising input costs, rather than targeting maximum production levels. It is not surprising, therefore, that the grass and legume seed sales have been declining in many countries, particularly in Europe, with, for example, the UK market having more than halved since 1980. Furthermore, grass and legume seed prices have not kept pace with world prices and yet the costs of seed production and distribution have been continually rising. The implication of this has been the progressive loss of grass and legume breeding programmes or their absorption into large conglomerates. This has possibly been most evident in Europe where many companies have either ended their forage programmes or linked or sold them into larger organisations that can operate on a more global scale.

The underlying problem has been that the link between reseeding costs and animal profitability is very poorly quantified. National and recommended lists successfully exclude below-standard performers and describe the relative ranking of cultivars for a number of useful performance characteristics. While undoubtedly the best cultivars get listed, failure to quantify precise animal production potential is a consequence of virtually infinite variations in end use and absence of exact scientifically validated specifications for optimum ruminant requirements.

For too long, this has been recognised as an important limitation of forage grass and legume VCU testing (Camlin 1997), without a satisfactory resolution having been found. It is notable that of the three cultivar testing functions, only VCU evaluation does not have an umbrella organisation to internationally harmonise testing procedures, agree standards and organise the coordinated research effort needed by industry. Without this additional level of harmonised regulatory control, agricultural forage breeding will struggle to achieve the incomes necessary for reinvestment in innovative research to the benefit of agriculture overall.

8 Concluding Overview

The PBR and seed testing schemes for grasses and legumes function as they were intended by successfully protecting and awarding breeders IPR and facilitating the distribution of high-quality, authenticated seed internationally. The VCU testing of amenity grasses has been equally successful in identifying elite cultivars that continue to fulfil the diverse needs of an exacting but profitable market sector. The VCU system for forage grasses and legumes has also successfully promoted breeding improvement, but into contracting markets with downward pressures on input

costs. While several challenges face the testing authorities, possibly the greatest is quantifying 'forage value'. This needs to quantify the benefits to farmers in terms of direct ruminant cash products and to government legislators in terms of environmental factors such as reduced carbon footprint of ruminant farming. This has yet to be adequately achieved and requires the testing authorities and breeders to collectively redress this knowledge void. It must be done without adding to testing costs or by unnecessarily overloading breeders with multiple additional performance criteria that together could impede rather than promote breeding progress.

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Future Developments and Uses

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1 Expected Changes in Grassland Management

All impacts to our environment, including agriculture and grassland management, are tied directly or indirectly to world population and its continued growth. It is estimated that it took the world until 1804 to sustain 1 billion people on the planet; then only another century to add 2 billion; then in less than a hundred years over 4 billion more have been added (Anonymous 2009). In 2006, world population was estimated to be increasing by an astonishing 211,000 persons per day and there is no reason to suspect this growth trend has abated.

These unprecedented changes in population growth are responsible for increasing pressure for producing more food on the world's arable, and now even marginal, crop land including intensively managed pastures as well as extensively managed grasslands and rangelands. Related pressures are the needs and wishes of the many diverse segments of this growing population. In fact, public demands on all grasslands, including amenity areas, are related to their multiple functions and values that range from fodder for both domestic and wild animals, to ensuring clean water sources, to an ability to sequester carbon and help clean the air, to protect soil from erosion, to protect animal and plant biodiversity and their habitats, to support tax income for rural communities, and to provide recreational opportunities and open space and improvement of quality of life (Peeters 2008).

Peeters (2008) reported that, historically, intensively managed grassland systems succeeded in increasing yield and quality of forages, and in turn, insured an increase in total production of animal food products. However, this process was accompanied by changes that included a large decrease in farmer population and an increase of farm size and a general modernization of grassland agriculture that used much more inputs than in the past such as nitrogen fertilizers, soil amendments, herbicides, irrigation, concentrate feed, and crops like maize. Investments were also made in buildings and machinery. All these changes induced enormous productivity gains whose benefits were largely transferred to the rest of society. The farming sector also provided the manpower that allowed other sectors of the economy to

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grow. These systems therefore provided safe food at a relatively low price and in a regular manner (food security) for the consumer. However, several unforeseen effects of these systems progressively appeared such as landscape changes, biodiversity reduction, pollution (especially runoff of nitrogen fertilizers and pesticides), erosion, misuses of natural resources, and degradation in product taste. In some areas, irrigated forage production competed with industry and urban areas for water use.

The benefits of breeding fodder crops and amenity grass varieties were important during this time due to the use of improved varieties to underpin these grassland systems and their direct and indirect products. For example, there are literally thousands of grass and legume varieties recognized by the commercial seed trade leading to the conclusion that the economic benefits from forage crop improvement were immense during this time of increased productivity of grassland areas (Bouton 2007a).

Policy responses to the overall problems were very diverse across all the continents (Peeters 2008). Current agro-environmental policies are focusing mainly on reducing nitrate and phosphate pollution, increasing biodiversity, and conserving or restoring landscapes. Support to the grower and to his income was used as a reward for their positive contribution to land and natural resources. More recently, policies are being developed on climate change mitigation. However, these policies raised many questions about funding efficiency. At present, it appears that budgets associated with these environmental policies remain small and probably ineffectual.

These policy responses are now causing political debates, and in some cases, new approaches for using both intensively and extensively managed grasslands – all against an environmental and social background that cannot accept risk and cannot even agree on real versus perceived risk. This complicated picture at the world's societal level leads one to hope that the best government policies will continue to evolve, and in turn, positively affect how all grasslands are managed and new varieties are developed.

2 Expected Changes in Forage Crops on Arable Land

Grasslands are normally relegated to the poorer soils and marginal lands and this trend will surely continue for most of the world. This model is especially true as population growth continues and good arable land is converted to cities, housing, and shopping malls. So, all cropping systems, whether based on row crops or fodder and amenity crops, are expanding in production to meet growing populations yet simultaneously moving onto more marginal land. It is just that the row crops will continue to occupy the best of the remaining land, while fodder and amenity crops will occupy the poorer land. It follows there will need to be new species utilized that are more adapted to marginal lands, and their accompanying abiotic and biotic stresses, or traits incorporated into existing popular species so they can be more productive in these lands, or both. Therefore, breeding and selection programs concentrating on abiotic and biotic stress tolerances are now important and will continue to grow in importance for traditional grassland uses.

There is probably a good case to be made that forage crops will also be used more in the future in multiple cropping systems on arable land as we continue to rediscover sustainable practices like crop rotation and green manure. Therefore, if nitrogen fertilization, and its negative impact on farm income and environmental sustainability, continues to be an important problem, then there will always be a use for growing a forage legume crop like alfalfa then rotating a row crop like corn into it. The corn/alfalfa rotation, so popular in North America during the time right after World War II, could become popular again due to its economic ability to reduce the need for expensive nitrogen fertilizer.

Another role is for intercropping systems, that is, growing legume-grass mixtures for sustainable animal production or forage crops between tree nurseries or orchards as a way to trap insects then spray them on the ground. Another option is using forage crops in wildlife management, which is actually becoming a big issue for both agro-tourism and establishing wildlife plots within forests for hunting.

Of course, all these types of mixed systems are very sustainable, have had a big part to play in the past, and should continue to do so in the future. The main thing here is these are old approaches and now there is interest in recycling them into our new management paradigms. This is true mainly because of a real need for more sustainable systems in the modern era.

3 New Uses of Forage Plant Products

As outlined above in Section 1, society's demands for grassland areas, as well as their future expectations, are high and possibly even contradictory. The biggest new use will therefore be the old use of producing animal products within an arena of good soil, land, and animal husbandry as well as continuing to supply amenity grasses for conservation and general-purpose turf. However, there are two emerging areas within these traditional systems that could add value in the near term. The first is the possibility of simultaneously using forages and amenity grasses as carbon sequestration crops and selling CO_2 credits from this land. In this regard, a significant, but variable soil carbon increase was reported when monitored across large acreage switchgrass (*Panicum virgatum* L.) fields (Liebig et al. 2008). Therefore, selling CO_2 credits seems a likely new use as it can be done with both the current perennial systems as well as newly planted areas. Second, the demand for identity preserved, "natural", "grass finished", or "organic", animal products is increasing as certification programs are now available to add value to the products. Although they will probably remain small as a percentage of overall food production, these markets provide high margin possibilities for forage/livestock producers.

The second new use is forage crops as cellulosic biofuel feedstocks. The main criteria for any biofuel crops are high yields achieved with low input costs in an environmental friendly and sustainable manner. By this definition, many high yielding, currently grown perennial forage crops are good candidates as biofuel crops especially if they can be delivered to a biorefinery as cheaply as possible (Bouton 2008).

To support this conclusion, an important recent study demonstrated that switchgrass grown as a biofuel feedstock on actual working farms was cost-effective from both an energy and an economic perspective (Schmer et al. 2008).

Biofuels, along with wind and solar power, are one of the new "green" technologies being considered to replace fossil fuel energy sources, but like everything in our modern culture, there are controversies and concerns. Two disparate controversies surrounding biofuels are the current use of food crops such as corn and sugarcane as the main feedstocks (food versus fuel argument), and second, that the main biofuel produced is mostly ethanol. For the second problem, conversion of a feedstock directly to synthetic diesel or gasoline would be more desirable to the oil and gas industry due to ethanol's high water requirements and the corrosive ability of this water to pipelines and some engines. However, it is difficult at this time to predict what bioprocess will succeed in the long run and breeders of biofuel crops will simply need to be alert to possibilities of breeding for traits that enhance the crop's use for that particular bioprocess. In the interim, the main trait is simply high yields with low production inputs achieved in an environmentally friendly manner.

For the food versus fuel problem, the good news is that based on current estimates, cellulosic feed stocks, such as perennial forage crops, are far better than grain crops as a biofuel feedstock. Cellulosic feed stocks are also estimated to be more CO_2 neutral and to produce five times more energy than corn grain and are intended to have a broader range of adaptability especially on poorer soils. This would also allow them to be grown in regions that cannot support large-scale grain production. The main feed stocks being considered are crop residues and perennial crops such as grasses, and trees, and animal manures and even municipal wastes (Bouton 2008). For the perennial grasses the main ones being investigated are switchgrass, giant miscanthus, and giant reed. However, in these early days while there are very few bioprocessing facilities, it is also important that these crops have alternate uses. The obvious alternate use would be as forage for livestock, and that is why switchgrass is a very good choice in North America. Traditional high yielding forages like bermudagrass, tall fescue, ryegrasses, red and white clover, and alfalfa also have potential based on this model. However, for biofuel, they will all have to meet the requirements of a low cost of delivered feed stock; possibly as low as US $50 per ton. Therefore, high yield per unit area and high yield per unit input are paramount and could be the greatest hurdle for growers of traditional forage crops to overcome.

4 Opportunities for the Application of New Technologies in Breeding

At the International Grassland Congress in New Zealand in 1993, the two main themes raised during forage improvement discussion section were (1) the current and future role of public plant breeding and how this role will be defined in the context of reduced funding and (2) the methods and cost associated with proper evaluation of a variety's merit (Bouton 1993). In the context of the release and

farmer use of new forage varieties, it was not clear in 1993 what the role of new technologies, especially biotechnologies, was going to be. In those days, biotechnologies had potential to add either novel variation not found in the available germplasm or speed up the selection process itself. However, participants of the practical plant improvement session did not deal with these two issues to any extent and the papers, posters, and discussion at that congress would lead to a conclusion that they did not view them a bottleneck larger than the evaluation process itself. Also, most problems associated with forage crops were believed to be governed by multiple genes which are not so easily manipulated with biotechnological techniques. Therefore, the conclusion at that time was biotechnologies still have great potential, but the lack of interest shown to them raised questions regarding their short-term impact.

Now move forward almost 15 years, and the term molecular breeding is used routinely and describes the application of genomic and transgenic biotechnologies in conjunction with traditional forage crop breeding (Bouton 2008). However, molecular breeding approaches are expensive and, in the case of transgenics, controversial requiring much planning and even partnerships or consortia with others to defray costs and overcome a "valley of death" for cultivar commercialization due to patenting and regulatory issues (Bouton 2008). Although alfalfa with the Roundup Ready® gene was briefly commercially available (only to be re-regulated), it is instructive that currently there are no transgenes deployed in any forage crop. The future of transgenics therefore appears to lie mainly in the hands of regulatory agencies and their ability to establish a fair process to evaluate real versus perceived risk. Even with these problems, transgenesis is now routine in many forage species (Wang et al. 2008), and value-added transgenes are being inserted into some of the main forage crops that have the potential to be commercialized if these inherent patenting and regulatory problems can be overcome (Smith et al. 2005, Wang et al. 2008). Finally, transformation programs are still viable for basic research purposes when used to create unique plants for documenting biochemical and physiological pathways (Bouton 2008).

There is less controversy, and greater application to variety development, associated with the application of genomic technologies to forage and amenity crop improvement. Using genomic technologies to assess and characterize genetic diversity (Kölliker et al. 2008) and to efficiently speed up the breeding process via marker-based selection (Brummer et al. 2007) has gained the most traction for application in crop improvement. Molecular markers to select for simple traits appear easy, but selection for complex traits governed by quantitative trait loci (QTL), especially those surrounding stress tolerances, has proved as difficult as it is important, and accurate phenotyping still remains the most difficult issue (Brummer and Casler 2008). Bernardo (2008) also concluded that finding QTL for complex traits is easy, but using them during selection is difficult. Although gain per cycle is currently not proven to be greater with marker-based selection than phenotypic selection, markers are showing that they can increase gain per year and per unit cost. As more marker data become available and phenotypic data less available, decisions should become more based on genotype than phenotype. Plant breeders then would

need to design marker-based breeding schemes that consider both the routine availability of marker data and the continuing challenges in obtaining good phenotypic information.

Bi-parental mating designs to detect QTL may actually reduce application, and there are few statistical methods available capable of handling polygenic traits. To overcome these problems, genomic selection (GS) is proposed as a way to predict the breeding values within elite breeding populations by analyzing both phenotypes and high-density marker scores (Heffner et al. 2009). The strengths of GS are avoidance of marker bias, a better ability to capture QTL with small effects, acceleration of breeding cycles, and better gain per unit time. However, it is its potential to reduce phenotyping that is most intriguing since phenotyping only needs to be used to update prediction models.

One of the most fascinating and potentially important research areas is microbial mutualists and their ability to add ecological fitness to their grass hosts (Bouton 2007b). This area's past was dominated by a geographic focus in New Zealand, USA, and Australia, due mainly to the economic impact of tall fescue and perennial ryegrass when infected with *Neotyphodium* fungal endophytes. There were many research and technological achievements reported for *Neoptyphodium* associations with commercialization of elite cultivars re-infected with "novel" (non-toxic) endophytes such as "AR1" and "MaxQ" as examples of positive on-farm impact. In the future, it is anticipated that host breeding, commercialization, and on-farm use of novel endophytes will increase including expansion into other grass species, and even cereal crops, sequencing and gene expression data will be available for the main fungal genomes, microarray, genomics, and transcriptome approaches will grow in importance, and examination of other possible bacterial and viral mutualists will proceed.

Finally, with the reduction in funding for public breeding programs, future resources are going to fewer and more traditional forage crops, and these crops are the ones that have greater economic value and are therefore more attractive to apply biotechnologies (Bouton 2007a). This has the potential to favor crops like alfalfa, perennial ryegrass, and white clover and penalize the other grasses and miscellaneous legumes. Even for crops with an identifiable path to market, this concentration of resources in fewer hands requires development of consortia where organizations can leverage their resources with others who possess complementary strengths.

5 The Impact of Climate Change on Forage Crops and Grassland

The American writer and humorist, Mark Twain, who when people asked if he believed in infant baptism, replied "believe in it, hell, I have seen it"! Well, most farmers since agriculture's beginnings would agree with using that same response for the question of do you believe in climate change? So, from a farmer's

perspective, climate change is nothing new as they have always seen extreme weather changes from year to year especially as it affected their crops. In other words, one year it could be extremely wet and cool, and the next year it could be extremely hot and dry so that one could set weather records in the same locale during a 12-month period. Although farmers normally talk about weather (short term) and not climate (long term), their immediate weather concerns make them extremely risk averse and normally reduce their long–term climate concerns due to immediate economic needs. If the prediction models of future climate change and global warming are as dire as predicted (Anonymous 2009), then what will separate the best farmers from the rest is adjustment to conditions before, but certainly as they change.

Predictions of climate change then represent a call for an "adjustment philosophy" for both farmers and researchers; if it gets hotter and drier, then plan for hotter and drier. From a breeding standpoint, programs developing new fodder and amenity crop varieties will need to concentrate on weather extremes that so often happen now on a year-to-year basis. Breeding programs concentrating on stress tolerances will continue to grow in importance. It is just for the future, the breeding strategies may be for even wider extremes especially when these weather extremes are combined with the movement of crops onto more marginal lands as outlined in Section 2. Therefore, many fodder crop and amenity grass breeders probably will not change the current strategy of breeding for extreme stress conditions, it is just that targeted traits for drought or heat or even cold tolerance will be for even wider extremes.

The need to develop crops for extreme climatic and soil conditions is also why breeders must use all available technologies such as microbial mutualists, but especially genomics and even transgenes, if they are to have a chance of succeeding with growing crops in these predicted harsh situations. This will require using progress in scientific knowledge at the molecular level in the most sustainable and safe way possible, so as to improve it's acceptance by the broad public. A good example is the potential to use drought transgenes like WXP1 (Wang et al. 2008) that would be stymied under the current regulatory climate. Our expanding world population wants more and safer food, grown on more marginal land, exposed to harsher climates, and all done with more attention to environment, but hesitates to accept some of the breeder's most powerful tools for achieving these milestones. This conundrum is also just another example of society's contradictory nature and unwillingness to accept even the slightest risk. Of course, the agricultural industry has done a poor job in laying out its case for how it is now achieving society's varied milestones via modern agricultural production that includes a dramatic increase in use of varieties containing biotech traits. It is now beginning to do this for the major row crops with a very recent report that defines a "sustainability index" developed from metrics around socioeconomic, health, and environmental outcomes (The Keystone Center 2009). These initial findings document substantial decreases in energy use, soil loss, water use, land use, and climate impact during the period 1987–2007 for crops like cotton. In fodder and amenity crops too, efforts like this will be indispensable for future discussions not only with policy makers but also with the society.

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Ryegrasses

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1 Introduction

Perennial ryegrass (*Lolium perenne* L.), Italian ryegrass (*Lolium multiflorum* Lam. ssp. *italicum* Volkart *ex* Schinz *et* Keller), Westerwolths ryegrass (*Lolium multiflorum* Lam. ssp. *multiflorum;* Hanelt and IPK 2001) and hybrid ryegrass (*Lolium boucheanum* Kunth) are the main forage grasses sown in northwest Europe, New Zealand, and in the temperate regions of Japan, Australia, South Africa and South America. Perennial ryegrass is also widely used in amenity grassland including sports turf. This is reflected in the number of varieties on the OECD list and in global seed production (Table 1; Nils Elmegaard, Danish Seed Council, pers.com.). *Lolium* species account for about 23% of the 52 million ha of grassland in Europe with perennial ryegrass being the most prevalent species. Since 2000 the EU-27 countries have produced, on average, 83,660 t perennial ryegrass and 39,010 t Italian/Westerwolths ryegrass seed per year. Ryegrass species differ in the proportion of growth produced from leaves or stems. In aftermath growth, Italian and Westerwolths ryegrasses mainly produce growth based on stems from reproductive tillers while perennial ryegrass has a higher proportion of leaf growth from non-reproductive tillers. The leaves and stems of ryegrasses are generally more digestible than in other grass species (Frame 1991).

1.1 Perennial Ryegrass

L. perenne is a perennial, highly tillering species which is productive over long growing seasons (at least from March to November in western Europe) and maintains yield well when grazed. It comprises a wide variety of types over a range of

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Table 1 Number of varieties on the OECD list and average annual seed production for *Lolium* species

Species	Common name	OECD Varieties 2007	Global seed production (t/year) 1998–2007
Lolium perenne L.	Perennial ryegrass	1,156	209,674
Lolium multiflorum Lam.	Italian and Westerwolths ryegrass	472	151,259
Lolium x boucheanum Kunth (*L. x hybridum* Hausskn.)	Hybrid ryegrass	110	6,923
x Festulolium spp.	Festulolium	19	1,179

heading dates from early April to late June with a large amount of genetic variation from a wide ecotypic range providing many adaptive phenotypes and a wealth of natural genetic resources.

1.2 Italian Ryegrass

L. multiflorum ssp. *italicum* is a biennial species with high digestibility and palatability and high value in livestock forage systems. Compared to perennial ryegrass it has a lower persistence and stress tolerance, but a higher yield potential and a faster ground cover (rapid emergence and excellent seedling vigour). Italian ryegrass generally develops very few seed heads in the seeding year and remains leafy throughout the entire season. It tends to be very shallow rooting and form a dense, fibrous mass about 5 cm deep which suggests that it does not tolerate dry conditions very well. Italian ryegrass is mainly used in short-term leys of 1 or 2 years for hay and silage making or as green manure and ground cover during the winter to prevent soil erosion and nutrient losses. It requires vernalisation and long days (thermo-photoperiodic requirement) for the production of reproductive tillers. As with perennial ryegrass, vegetative propagation of plants by separation of tillers is possible.

1.3 Westerwolths Ryegrass

Truly annual Westerwolths ryegrass (*Lolium multiflorum* ssp. *multiflorum*) is a subspecies which sets seed in the year of sowing. Westerwolths tends to be taller than Italian ryegrass and is a leafy highly tillering grass of high palatability and digestibility. When spring sown under continental conditions, Westerwolths ryegrass will produce high first cut silage yields in the summer. It will then continue

growing to give further silage harvests until winter, when it will normally die out. Following high yields in the seeding year, persistence declines rapidly in the first winter whereas Italian ryegrass will persist well through a second winter.

Westerwolths ryegrass is of importance in the southeast of North America where it is overseeded as a winter annual into permanent warm season pastures on more than 1.3 million ha annually (van Santen and Bergtold 2007). In South America it is used in areas of Argentina, Chile, Uruguay and some parts of Brazil. It is an important forage crop in South Africa and is also used in New Zealand and South Australia. In Asia, Westerwolths ryegrass is common in Japan and China as well as in India. In Europe, the grass is used in the Mediterranean region and in the temperate areas of central Europe. Over 80% of Westerwolths in the world is sown in autumn and during winter and is followed by a summer arable crop in spring.

2 Origin and Systematics

2.1 Ryegrass Origins and Spread

The genus *Lolium* is based on diploid species with 7 pairs of chromosomes which form part of a *Lolium/Festuca* polyploid complex. Many species show some interfertility although two main groups can be distinguished based on pollination behaviour and ease of hybridisation. One group comprises inbreeding annual species commonly found as weeds of arable crops including *L. temulentum* L. (found in wheat and barley fields), *L. remotum* Schrank (known as a weed in flax) and *L. persicum* (found in cereal fields in West Asia), *L. loliaceum* Hand.-Maz and *L. canariense*. Based on morphological traits, Terrell (1968) suggested that *L. persicum* and *L. temulentum* are derived from the same basic stock. With improved arable crop cultivation and enhanced seed cleaning techniques the distribution of these weedy species is decreasing (Hubbard 1954) and they are now only rarely found on waste-land and rubbish dumps. *L. loliaceum* and *L. canariense* are found mainly on poor land and in maritime conditions (Loos 1994).

The second group of ryegrasses comprises outbreeding species including Westerwolths, Italian and perennial ryegrasses which are the most economically important *Lolium* species in Europe. Italian ryegrass contains biennial and weakly perennial forms while perennial ryegrass has a range from near annual to strongly perennial types. *L. rigidum* Gaud. is a winter annual originating from the Mediterranean region and is used as a cultivated fodder crop in some areas (e.g. Australia), but not in Europe although it may become more important in drier areas with climate change.

The Mediterranean basin is the likely origin of ryegrasses which share a high degree of genome ancestry with cereal species of Eurasian origin, such as rice, wheat and barley (Kellog 2001). The domestication of these grasses is associated with the emergence of primitive agriculture in the fertile crescent of the Middle East about 10,000 years ago and its subsequent expansion into west and north Europe

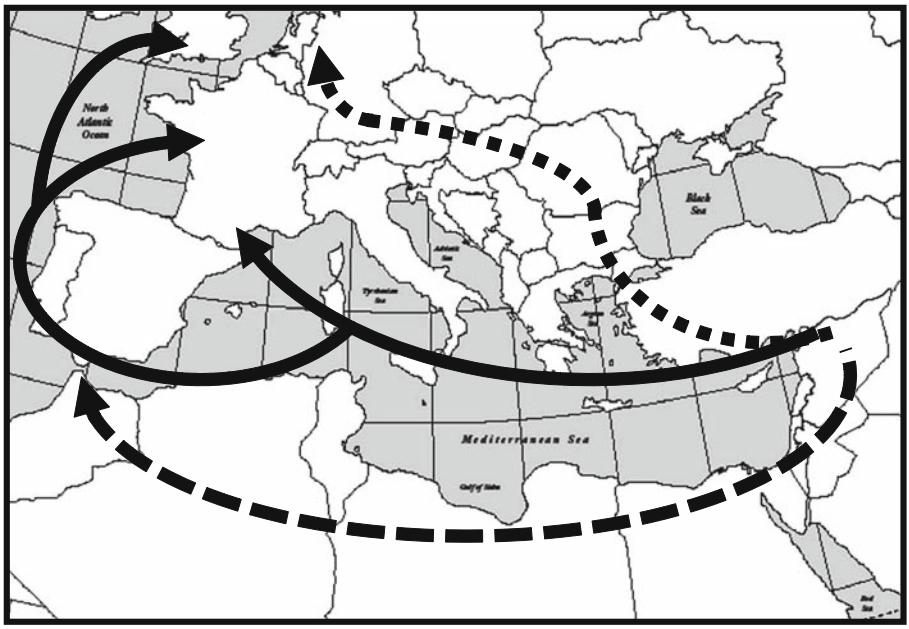

Fig. 1 Possible pathways for the spread of ryegrasses from the fertile crescent in the Eastern Mediterranean (after Balfourier 2000). —►, Mediterranean movement; - - ►, North African continental route; · · · ►, Danubian route

(Balfourier 2000, Figure 1). Ryegrasses were probably spread as weeds of cereal crops by migrating farmers. In Jordan and Syria weeds in wheat fields are known as 'zawan' in Arabic (Musselman 2000). This includes *Lolium temulentum* L. which has a life cycle similar to wheat and is also known in Greek as zizanion which is translated as tares or darnel in the bible.

2.2 The Domestication of Ryegrasses

2.2.1 Perennial Ryegrass

The earliest reference to the use of grass seed for the deliberate conversion of arable land into grassland was in 1677 in the UK with the sowing of 'Ray-grass or *Gramen Loliaceum*' (Jenkin 1949). According to Stapledon and Davies (1941), it was not until after 1874 that land was sown down to 'permanent grass' on a grand scale and that farmers began to gain experience in seeds mixtures, and had opportunities for noting the stages of development in long-duration swards. By 1910, the grass-clover ley was well established within a rotational system where 2–3 year old leys were ploughed up to provide sufficient nutrients for two or even three good cereal crops of wheat, oats or barley. This system recognised the dual aspect of grass-clover

swards in terms of a cheap feed for livestock and the promotion of soil fertility. In the 1930 s new pasture strains of perennial ryegrass, such as Aberystwyth S. 23 were used with white clover in the rapid development of swards of the highest grazing value. Differences between 'hay' and 'pasture' strains of grasses were recognised with 'hay' types being more stemmy, shorter lived and earlier to start growth in the spring compared to 'pasture' strains. 'Hay' strains, as long as they persisted, were particularly valuable for early spring grazing during the earlier years of a ley while the 'pasture' strains were of especial value for season end and winter grazing in longer term leys (Stapledon and Davies 1941).

2.2.2 Italian Ryegrass

As its former scientific name *Lolium italicum* Br. and its common names in most European languages imply, this species can be safely assumed to be of Italian origin. Beddows (1973) suggested that it was unknown to botanists or agriculturists in the UK until the early nineteenth century and that the earlier mention of *Lolium* with awns or 'beards' by Ray (1724) and Martyn (1792) referred to *Lolium temulentum* and not to *Lolium multiflorum*.

2.2.3 Westerwolths Ryegrass

Selection work by grassland farmers in the late nineteenth century marks the beginning of a highly successful subspecies of *Lolium multiflorum*. According to de Haan (1955), the annual 'Westerwolths' form of *Lolium multiflorum* came about by repeated harvesting of fields sown with Italian ryegrass in the spring in south-east Groningen in the Netherlands. The first trials of annual ryegrass under the name 'Westerwolths ryegrass' were reported in 1901–1902 at the Dutch state seed testing station in Wageningen and seed exports are known to have gone from the Netherlands to many parts of the world. In Germany, Westerwolths landraces were first certified in 1925 and by 1936 N.F.G., a predecessor of Euro Grass Breeding, was involved in the systematic breeding of annual ryegrass.

Whether or not farmers in South America, where annual forms of Italian ryegrass have been known for a long time, based their seed stocks on Dutch imports or selected independently for annuality in Italian ryegrass remains an open question. In the late 1940 s systematic selection started at La Estanzuela (Uruguay) where the first variety produced (LE284) was early flowering and had good winter growth in that area. In the USA LE284 served as the ancestor of the variety 'Gulf' released in 1958. In Florida breeding work started in the 1950 s using material from local ecotypes, domestic varieties and plant introductions (Quesenberry 2003).

2.3 Ryegrass Species Taxonomy

Perennial, Italian and Westerwolths ryegrasses have 7 pairs of chromosomes ($2n = 2$ x $= 14$) and a relatively large genome size (1 C $= 2000$ Mb) (Hutchinson

et al. 1979). Although these species are naturally diploid, tetraploids can be induced relatively easily. The species are natural cross-pollinators with a high degree of self-incompatibility controlled by 2 to 3 loci with many alleles. Fearon et al. (1983) showed that *L. multiflorum* was no less self-incompatible than *L. perenne* and that at least 40 different alleles are likely to be present at both the S and Z loci. However, some pseudo-self-compatibility can allow self-pollination to produce a small amount of selfed seeds. Italian ryegrass and perennial ryegrass can be distinguished by inspecting the leaves (folded in the bud for *L. perenne*, while rolled for *L. multiflorum*) or the seed (awnless spikelets for *L. perenne,* while with awn for *L. multiflorum*). Presence (*L. multiflorum* and *L. boucheanum*) or absence (*L. perenne*) of UV fluorescence in primary seedling roots can also be a distinguishing feature, although *L. perenne* varieties may contain a small percentage of fluorescent roots.

Despite their morphological differences, *L. perenne* and *L. multiflorum* (including Westerwolths) cross easily. The resulting hybrid, *L. x boucheanum* Kunth. (also called *L. x hybridum* Hausskn.), is fertile and is intermediate in terms of persistence and yield. Terrell (1968) argued that there is no evidence that Westerwolths ryegrass differs from *L. multiflorum* in other than minor ways and concluded that it should be considered as a cultivar of *L. multiflorum*. However, Westerwolths ryegrass is clearly distinguishable from Italian ryegrass by complete flowering in the year of sowing and there is emerging evidence of differences in DNA content. Thus Westerwolths is clearly more than a cultivar of Italian ryegrass and the term 'subspecies' is probably more correct. This view is substantiated by Hanelt and IPK (2001) who classified Westerwolths ryegrass as *Lolium multiflorum* ssp. *multiflorum*. In view of the world-wide commercial importance of Westerwolths ryegrass some clarification is necessary with regard to misuse of its common name in Australia. It is unfortunate that *Lolium rigidum* Gaud. is sometimes called 'annual ryegrass' in Australia rather than Wimmera ryegrass (Oram 1990). Consequently, health problems in cattle due to *L. rigidum* fodder infected with the bacterium *Rathayibacter toxicus* was named annual ryegrass toxicity (ARGT). However this is not associated with *Lolium multiflorum* including Westerwolths ryegrass.

3 Varieties and Varietal Groups

In 2009 the European variety catalogue lists about 963 *Lolium perenne* varieties (http://ec.europa.eu/food/plant/propagation/catalogues/). Included among these varieties are turf as well as forage types and in many cases it is not possible to distinguish between these two groups. There are 379 *L. multiflorum* varieties listed in the 2009 European catalogue and the 2008 OECD list names 472 *L. multiflorum* varieties eligible for seed certification. Thus only around 100 varieties are listed from non-European countries such as Japan, New Zealand, South Africa and Brazil with the USA, Australia, Argentina, Russia and Uruguay each having less than 15 varieties listed. The 2009 European variety catalogue list includes about 150 Westerwolths varieties. However in many countries it is not possible to distinguish between Italian and Westerwolths ryegrass as both types are included as annual ryegrass.

3.1 Grouping of Varieties

In general, all *Lolium* species can be grouped as diploids or tetraploids. During the last 50 years the proportion of listed tetraploid varieties has increased steadily. A comparison of the proportion of tetraploid varieties used in the Netherlands, Germany, France and the UK between the early 1980 s and 2007 is shown in Table 2.

Table 2 Changes in the proportion (%) of tetraploid ryegrass varieties since the 1980 s in different countries

Country	Year	Perennial	Italian	Westerwolths
The Netherlands	1984	20	47	46
	2007	34	60	*
Germany	1983	12	56	50
	2007	50	59	50
France	1983	7	50	60
	2007	58	46	48
United Kingdom	1984	15	39	40
	2008	41	36	*

(* In the Netherlands Westerwolths ryegrass is no longer listed and in the UK it is no longer differentiated from Italian ryegrass)

Whereas in the 1980 s the proportion of tetraploids in Italian and Westerwolths ryegrass varieties was about 50%, the proportion in perennial ryegrass was relatively low (from 7% in France to 20% in the Netherlands). In the following 20 years the proportion of the perennial tetraploids increased in all countries. The most drastic change was in France where it increased from 7 to 58% in 2007. The proportion of Italian ryegrass tetraploids has remained more or less stable. Chromosome-doubled tetraploid ryegrasses tend to have larger leaves and tillers compared to diploids and produce herbage with a lower dry matter content and more open swards. Tetraploid perennial ryegrasses can be very high yielding with good sugar contents and the open swards prevent damage from snow mould. They tend to have a tall upright growth habit that promotes high intakes when grazed but are normally sown in mixtures with diploids to increase sward density and trampling damage resistance.

3.1.1 Perennial Ryegrass

Management trials of perennial ryegrass varieties are usually carried out in three maturity groups – early, medium and late. However when comparing varieties for farming use, they are best regarded as existing in a continuum that extends from the earliest maturing varieties heading around late April to early May to the latest maturing varieties heading around mid to late June. Diploid perennial ryegrass varieties are long-lived and form swards of good density, producing a relatively high tolerance of abiotic and biotic environmental stresses. They are well suited to a wide range of managements and the appropriate selection of varieties allows for the

production of high silage yields or the maintenance of high grazing outputs throughout the growing season. Seasonal yield distribution varies from the earliest to the latest maturing varieties with increased early season yields from the earliest varieties and increased summer production with the later heading varieties. Depending on local environmental conditions 'Spring' growth generally occurs up to the end of April, 'Early Summer' growth to the end of July, 'Late Summer' to the end of September and the 'Autumn' period generally ends in early November. The first silage cuts are normally completed by mid-May for the early types, the end of May for the intermediate types and during early June for the late varieties. This gives a 3- to 4-week spread in most years which is maintained to the end of the third cut, resulting in different periods of 'aftermath' growth.

3.1.2 Italian Ryegrass

Italian ryegrass varieties show little differentiation in maturity date. A narrow (2 weeks) range in heading date contrasts strongly with a much broader range found in perennial ryegrass (at least 6 weeks). Italian ryegrasses are generally higher yielding than perennial ryegrasses but are shorter lived and used mainly for silage with some limited grazing. Higher density varieties may be more resistant to trampling but none form dense swards. Good growth is usually provided by the end of March ('Spring Grazing') followed by two high yielding silage cuts completed by mid-July, to leave the option of further cuts or aftermath grazing.

3.1.3 Westerwolths Ryegrass

In terms of agronomic relevance the differentiation between varieties of the multicut or maincrop type with good regrowth and the one-cut or catch crop type with poor regrowth is of high importance. Physiological-genetical research during the last few decades has found day length sensitivity to be responsible for this differentiation. With increasing day length, as is found in the temperate zones of Europe, Westerwolths ryegrass switches completely from a vegetative to a generative state. In spring it will head 6–8 weeks after sowing. When sown in summer, day length sensitive types will not switch completely from the vegetative to the generative state. Yamaguchi and Suzuki (1985) showed that under long day conditions (24 hours daylight) 48 populations headed within 5 days whereas under short day conditions (13 hours daylight) there was a difference of 87 days between the earliest and latest heading populations. Feuerstein, Marum and Stewart (unpublished) observed heading dates for 30 accessions from all over the world after spring sowing in 1990 at four locations with very different latitudes (Table 3).

The extremely rapid day length change from 12 to almost 24 hours during the first half of the growing season in Norway caused the earliest and the latest varieties to head within 11 days. In regions with less pronounced day length changes and generally shorter days, such as southern France and New Zealand, the difference in heading date was 50 days. In Germany, which is intermediate in day length, the

Table 3 Variation in heading date in a world collection of Westerwolths ryegrass at different latitudes

		Heading date (days after sowing)			
Location	Latitude	Mean	Minimum	Maximum	Difference
Norway Ås	59° N	72	67	78	11
Germany Hof Steimke	53° N	80	74	93	19
France Mauguio	44° N	73	53	103	50
New Zealand Ceres Farm	44° S	71	52	102	50

difference was 19 days. While sensitivity to changes in day length is indispensable for multicut varieties, insensitivity is vital for summer catch crop cultivation. When sown in a central European summer, day length insensitive forms produce about 50% higher dry matter yields but a reduced capacity for regrowth compared to day length sensitive forms. In temperate areas where Westerwolths ryegrass is often used as a catch crop after barley to fill forage gaps, it is desirable that the grass does not persist after harvest and day length insensitive varieties with poor regrowth are preferred.

3.1.4 Hybrid Ryegrasses

Italian ryegrass has good establishment and considerable growth potential, particularly in the spring and early summer. However, Italian ryegrass continues to produce a high proportion of stem in the aftermath growth, which has a lower digestibility than leafy material. Italian ryegrass also has poor persistence and is therefore suitable only for short-term (2 to maximum 3 years) leys. Perennial ryegrass swards generally have higher leaf content and offer good grazing quality throughout the season. They are more persistent than Italian ryegrass, although they may not produce high yields, especially if nitrogen fertiliser inputs are low. By crossing Italian and perennial ryegrasses, hybrid varieties can be produced that combine useful characteristics from both parental species. Tetraploid hybrid varieties outnumber diploid hybrid varieties and the total area of hybrid ryegrass seed production in the EU-27 is 6071 ha (2.5% of the total seed crop area).

The seasonal yield distribution of hybrid varieties is strongly influenced by the differing seasonal growth characteristics of the Italian and perennial parents. Some hybrid ryegrass varieties strongly express perennial characteristics while others express their Italian parentage more. ‘Italian-like’ hybrids have the highest yields but lowest sward densities, whereas ‘perennial-like’ hybrids can persist for up to 5 years if carefully managed. Reciprocal crosses indicate that this is not simply a maternal effect. Varieties achieving both high yield and density are obviously highly valued.

4 Genetic Resources and Utilisation

Although perennial ryegrass has its primary centre of origin in the European-Siberian region of diversity (Zeven and de Wet 1982), more intensive fodder production is reducing the biodiversity present in many grasslands. To counteract this genetic erosion, a wide range of genetic resources have been collected and are maintained by European gene banks for present and future use (Marum et al. 1998). Since the beginning of grass breeding activities in the 1920 s, breeders have made their own collection trips, and they partly still do so. For many decades new varieties were developed directly from ecotypes. Perennial ryegrass ecotypes from north and south Europe have been crossed to produce valuable gene pools for improving seasonal yield and nitrogen use efficiency (Wilkins and Lovatt 1989).

Bolaric et al. (2005) investigated molecular variation and population structure in 22 cultivars of perennial ryegrass, mainly of European origin, using random amplified polymorphic DNA (RAPD) markers. Analysis of molecular variance (AMOVA) revealed much larger amounts of genetic variation within cultivars (66%) than between them (34%). This was confirmed by Calsyn et al. (2005) who analysed a subset of 80 accessions from the ECPGR core collection of perennial ryegrass using AFLP markers. Only 3% of the total variation in the dataset could be attributed to differences between geographical locations, 4% of the variation corresponded to differences between accessions (within locations), but the major part of the variation (94%) was due to differences within populations. Viera et al. (2004) studied the genetic structure of *L. multiflorum* using RAPDs in four populations from South America. They too found wide genetic diversity within populations and small diversity between populations. Peter-Schmid et al. (2008a) studied genetic diversity within specific habitats for *Festuca pratensis* Huds and *L. multiflorum* using microsatellites (SSRs). They found larger within-population variation for *L. multiflorum* (97%) than for *F. pratensis* (93%) and differences between Swiss ecotype populations and varieties were small. However, based on phenotypic data, clear differences were observed (Peter-Schmid et al. 2008b).

The earliest documented collection of genetic resources of Westerwolths ryegrass was undertaken by Dobimar von Kameke in Asia Minor in 1929, followed up by systematic breeding work which led to the annual ryegrass variety 'Einjähriges Weidelgras v. Kameke' being registered in Germany in 1936. Due to some taxonomic uncertainty it is virtually impossible to get a clear estimate of how many accessions of Westerwolths ryegrass are available in the databases of the world's gene banks. The largest database for *Lolium* is the European Central *Lolium* database from the ECPGR hosted at IBERS, Aberystwyth (http://www.igergru.ibers.aber.ac.uk/). This database has 1,255 *L. multiflorum* accessions from 30 countries. It is only possible to separate out some well known Westerwolths varieties, but depending on the donor they are described as cv. *westerwoldicum* (21), ssp. *gaudini* (3), ssp. *italicum* (97) or ssp. *multiflorum* (2). In Germany, the GBIS (Genbank Informations System, Gatersleben) contains 242 accessions of *L. multiflorum* but again it is not possible to distinguish between different types of *L. multiflorum*. The same is true for the National Plant Germplasm

System (GRIN) of the USDA/ARS where 194 *L. multiflorum* accessions are listed. Although it is not often possible to identify Westerwolths genetic resources in gene banks, it is known (e.g. Quesenberry 2003) that ecotypes or landraces have been used to produce varieties.

5 Major Breeding Achievements

5.1 Trait Improvement

In the early years of grass breeding the main aim was to improve persistency and yield (Lütke Entrup 2008). Genetic gains in dry matter yield of ryegrasses over the past 50 years have been estimated at between 0.2 and 0.9% pa (Wilkins and Humphreys 2003) although in some environments much smaller gains are evident (van Santen and Bergtold 2007). Much of the spectacular improvement in the grain yield of cereal crops due to plant breeding in the twentieth century was achieved by increasing the proportion of plant biomass allocated to grain. Changes in harvest index are limited in forages because all the above-ground biomass is harvested and an increased shoot/root ratio is potentially undesirable due to possible loss of efficiency in the uptake of water and plant nutrients. Current ryegrass varieties can produce average annual dry matter yields of 17 t/ha in Europe and it may be possible through conventional breeding to reach 25 t/ha in optimum environments. The theoretical maximum annual yield potential of temperate grasses in the UK is estimated to be 29 t/ha. Forage maize (a 'tropical' grass which fixes carbon through C4 rather than the C3 photosynthesis found in most native European temperate grasses) currently produces up to 19 t/ha in the UK but only with fairly high nitrogen inputs.

In more extensive sustainable grasslands, yield per se has less priority compared to traits associated with nutritional value, such as digestibility. Improving nutritive quality in forage species has benefited from advances in techniques to measure digestibility in the laboratory and developments in near-infrared reflectance spectroscopy (NIRS) (Brown et al. 1990). Many forage species are relatively undeveloped in terms of nutritive quality and breeders are challenged with improving digestible energy and protein without reducing environmental stress tolerance and resistance to pests and diseases. Gilliland (2007) reported that a single unit increase in digestibility (DMD) can increase dry matter intake by 0.2 kg/cow/day and produce an increase in milk yield of around 0.4 kg/cow/day. For a 6-unit increase in silage digestibility, milk production increases by about 2 kg/cow/day and milk protein rises by 0.1%/cow/day.

The DMD of the leaf lamina is generally higher than that of flowering stems comprising leaf sheath, true stem and developing inflorescence. Stem digestibility is more important in determining the DMD of hay and silage cuts, when up to 70% of the herbage consists of stem, than DMD under grazing or monthly cutting, when most of the herbage harvested consists of leaf lamina. Increasing the dry matter content of herbage without reducing digestibility can improve the voluntary intake

as well as the fermentation of silage. Diploid ryegrasses tend to have lower water content than tetraploids and wilt more rapidly after cutting (Baert 1994).

Significant improvement in the energy value of grasses has been achieved by increasing sugar content (Wilkins and Humphreys 2003). Feeding high-sugar grasses, which accumulate 10–15% more sugars in leaves and stems, has improved milk production and milk protein content in dairy cows as well as increased liveweight gain in beef and sheep. Wilkins and Lovatt (2004) showed that average water-soluble carbohydrate (WSC) concentration has increased by 37 g/kg in *L. perenne* varieties bred between 1991 and 2000 whilst yield increased by 13%. They also reported increases of 35 g/kg in WSC and 10% in yield for tetraploid hybrid ryegrasses developed between 1988 and 1994, in addition to increases in persistency, nitrogen use efficiency and length of growing season.

A major source of alkaloid toxins in ryegrasses is caused by *Neotyphodium* endophytic fungi found mainly in the leaf sheaths of ryegrasses although they propagate by growing up the flowering stems to infest seed. Some alkaloids produced through endophyte infection (e.g. peramine) deter insect pests such as Argentine stem weevil while others (e.g. lolitrem B and ergovaline) cause loss of muscular coordination, ryegrass staggers, in grazing animals. There has been considerable progress in developing 'safe' strains of endophyte which deter insect attack but do not produce the alkaloids that are the main causes of toxicity to grazing animals (Easton 2007).

Rusts (*Puccinia* species) can be particularly damaging to ryegrass swards since they reduce herbage sugar content and digestibility as well as yield (Potter 1987). Both quantitative and qualitative resistance to crown rust has been found in ryegrasses (Roderick et al. 2003) and natural resistance from diverse genetic resources has been transferred into breeding populations using recurrent phenotypic selection (Figure 2). This resistance appears quite durable with the EUCARPIA multisite

Fig. 2 Individual-plant selection for crown rust resistance is often easy to perform in spaced-plant nurseries because differences can be assessed easily and reliably after natural infection, such as shown here with perennial ryegrass (Photo U. Feuerstein)

trials indicating no change in the ranking of varieties over 10 years (Schubiger et al. 2003, 2007).

5.2 Chromosome Doubling

One of the earliest 'novel' achievements of plant breeders was to double chromosome number in plant cells. In the early twentieth century different scientists tried methods such as low temperature treatment and interspecific grafting to achieve this. Finally the work of Blakeslee and Avery (1937), who described the first use of colchicine for tetraploidisation, led to significant progress. Success with this novel treatment proved to be repeatable and colchicine began to be widely used. The first tetraploidisation in ryegrass with colchicine was done by Meyers (1939) and the first variety (Probstheidaer tetraploids) was released in East Germany in the early 1950s. Wit (1958, 1959) was the first in the Netherlands to treat breeding material systematically and supply valuable tetraploid resources to commercial breeders. Similar work was also carried out by Hertzsch in Germany. Various techniques to improve the consistency of chromosome doubling were described in the 1950 s. In most cases germinating seed was dipped into a 0.2–0.5% colchicine solution for 1–3 hours which worked quite well but with a high rate of mortality. Better results were achieved by adding Tween 80 (Polyoxyethylene-sorbitan-monolaurate) and Dimethylsulfoxide (DMSO) (Morgan 1976). However the success rate of treatments remained genotype dependent with survival varying between 0 and 25%. Selection of genotypes surviving colchicine treatment may have both negative and positive effects on plant growth and development (Hague and Jones 1987). Following successful tetraploidisation, the vegetative tissue of many plants may contain a mixture of diploid and tetraploid cells and analysis of first generation plants may be misleading. It is better to examine progeny from harvested seed for ploidy. Until the late 1980s this was mainly done by counting chromosomes under the microscopes. With the introduction of the Partec Cell analyser CA-II 1987 the detection of tetraploid plants based on DNA content per cell became much easier. Today flow cytometry (FCM) is a reliable and quick technique and is the method of choice for most grass breeders. Thousand seed weight of tetraploids is about 40% higher than that of diploids and can serve as an easy-to-determine alternative when no laboratory access is available.

Several comparisons have been made between diploid and tetraploid ryegrasses (e.g. van Bogaert 1975, Feuerstein 1989). The general conclusion is that the yield potential of tetraploid compared to diploid varieties increases from perennial ryegrass to Italian ryegrass followed by Westerwolths ryegrass indicating that advantages are greater in shorter-lived species. In single cut Westerwolths ryegrass, the advantage of tetraploidy, possibly due to a larger seed size, is greater than in more perennial types and tetraploid Westerwolths and Italian varieties were the first to be marketed. Other advantages of tetraploid compared to diploid ryegrasses have been identified, such as higher crown rust and snow mould resistance.

Studies carried out on hybrid, perennial and Westerwolths ryegrasses (Mansat et al. 1966, Hague and Jones 1987, Feuerstein 1989) indicate that tetraploid

Table 4 Difference and relation between isogenic di- and tetraploid Westerwolths ryegrass (Feuerstein 1989)

Character	Diploid	Tetraploid	Sig.	r^2	Sig.
Tiller number (n)	10.5	7.8	**	0.37	**
Ear number (n)	109.9	68	**	0.19	n.s.
Leaf width (mm)	7	7.5	*	0.37	**
Leaf length (cm)	17.6	16.8	n.s.	0.46	**
Tiller length (cm)	70.1	63.6	**	0.3	**
Tiller diameter (mm)	1.4	1.6	**	0.18	n.s.
Ear length (cm)	18.9	20	*	0.52	***
Spike density (n/dm)	11.1	11.7	n.s.	0.44	***
Florets per spike (n)	11.7	10.3	**	0.2	n.s.
Pollen diameter (μm)	38.6	44.3	**	0.02	n.s.
1000 seed weight (g)	3.3	4.1	**	0.03	n.s.
Heading date (days)	60.7	65.2	**	0.72	***
Regrowth (score)	3.5	3.2	n.s.	0.29	**

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

characteristics very much depend on diploid origins. Feuerstein (1989) showed that it is simple to produce an early tetraploid from an early diploid Westerwolths ryegrass but much more difficult to transfer flowering head number from the diploid to the tetraploid level (Table 4).

Carlier (1974) compared the fodder quality of diploid and tetraploid Italian ryegrass. He found that tetraploid 'Meritra' had higher fresh yield but no higher dry matter yield than diploid 'Lemtal'. 'Meritra' had, on average, a lower dry matter content but a higher crude protein, ash and water-soluble carbohydrates content; but no significant difference in digestibility was found. Breeding tetraploid *L. multiflorum* has been successful in increasing yield, digestibility and persistency. Tetraploid varieties on the Swiss recommended list of 2009/10 have higher digestibility than diploid varieties but still have lower persistency and dry matter yield. Again tetraploid varieties appear to have higher resistance to leaf diseases and there is evidence that tetraploid *L. multiflorum* varieties have higher WSC content than diploids (Carlier 1974, ILVO unpublished data).

5.2.1 The Use of Tetraploidy in Species Hybrids

Chromosome doubling of parents by colchicine has been used to produce tetraploid *Lolium* hybrid varieties, and to facilitate introgression between *Lolium* species and from *Festuca* species into *Lolium*. Stable *Lolium* hybrid varieties have been produced by crossing chromosome-doubled *L. multiflorum* and *L. perenne* breeding lines (Jones and Humphreys 1993). Such hybrids combine the beneficial characteristics from *L. multiflorum* (e.g. rapid establishment and early growth characteristics) and *L. perenne* (persistency, stress tolerance and leafiness).

Because of genome stability problems in amphiploid hybrids, more emphasis is now placed on using DNA markers to monitor the introgression of genes associated

with specific traits. This includes improved early season growth in perennial ryegrass from Italian ryegrass; improved persistency in Italian ryegrass from perennial ryegrass; and improved drought tolerance, nitrogen use efficiency (NUE) and winter survival in ryegrasses from fescues (Humphreys et al. 2006). A gene responsible for reduced rates of chlorophyll degradation and protein loss during leaf senescence has been transferred from *F. pratensis* into *L. perenne* (Thorogood 1996).

5.3 Other Breeding Achievements

Van Wijk and Reheul (1991) reported an average yield improvement of 0.2% per year for newly listed Italian ryegrass varieties while van Santen and Bergtold (2007) indicated no consistent yield improvement in *L. multiflorum* since 1990 in the USA. In the first half of the twentieth century Westerwolths ryegrass breeding programmes concentrated on multicut varieties which produced good yields in the first cut after spring sowing followed by a couple of later cuts. As patterns of utilisation have changed over time, the date of the first cut has advanced and a good Westerwolths multicut variety is now expected to provide at least six productive cuts in a year.

5.3.1 Development of a New Catch Crop

Around 1960 the need for a completely new type of variety which could be sown as a catch crop after an early main crop in temperate regions was proposed by Clemens Stahl of Deutsche Saatveredelung (DSV). By means of several cycles of recurrent selection Stahl created a variety that was no longer day length sensitive, produced ample stems and heads after summer sowing but had poor regrowth. This new catch crop type variety was released as ‘Lirasand’ in 1974 and is still in the market. It became the most successful Westerwolths ryegrass variety in the world with total seed sales having risen above 100,000 t. Catch crop types can be used for grazing, and cutting for fresh indoor feeding or ensiling. Feuerstein (1989) treated about 800 seedlings from ‘Lirasand’ with colchicine and obtained, after three cycles of open pollination, 21 breeding lines for testing at two locations under main crop and catch crop management (Table 5). The three best breeding lines were multiplied in 1989 and after VCU-testing in Germany they were listed as ‘Liquattro’, ‘Litoro’ and ‘Livanti’ in 1993. These three tetraploid varieties were the first pure catch crop types to be released and provided new options for forage production.

5.3.2 Breeding for the US Subtropical Region

At the time when Westerwolths ryegrass was developed for catch cropping in central and western Europe, ‘common ryegrass’ was turned into annual ryegrass in the southern part of USA. After 29 years of natural selection the variety ‘Marshall’ was

Table 5 Dry matter yield of the first cut after spring sowing and summer sowing of 21 tetraploid breeding lines from the Westerwolths ryegrass variety Lirasand tested at two locations in 1988

	Main crop (spring sown)		Catch crop (summer sown)	
	g/m^2	(relative value)	g/m^2	(relative value)
Lirasand (2x)	345	(100)	298	(100)
Aubade (4x)	384	(111)	293	(98)
Twentyone tetraploid breeding lines derived from the variety Lirasand				
Average (4x)	360	(104)	302	(101)
Min	336	(97)	256	(86)
Max	383	(111)	334	(112)

released in 1980 (Arnold et al. 1981). In order to improve its crown rust resistance, 'Marshall' was subjected to four cycles of phenotypic recurrent selection leading to the variety 'Surrey'. 'Surrey' exhibited a huge improvement in crown rust resistance over 'Marshall' (Prine 1996). Based on 'Surrey', further cycles of selection for disease resistance, followed by tetraploidisation and further selection cycles resulted in 'Jumbo', released in 1999 (Prine et al. 2002). Further breeding work, involving 'Jumbo' and selections out of 'TAM90', led to the variety 'TAMTBO' which was released in 2006 (Nelson et al. 2007).

6 Specific Goals in Current Breeding

6.1 Breeding Goals in Perennial Ryegrass

6.1.1 Yield

Dry matter production per unit of nitrogen input continues to be an important primary objective in making the most efficient use of land resources to meet increasing global demands for food, feed, fibre and fuel. Baert et al. (2007) compared the yield of 24 varieties of perennial ryegrass at 450 and 270 kg N/ha/year. The ranking of varieties for DM yield was quite similar at both N application levels. The same trend was observed with 24 F_2 populations at 370 and 260 kg N/ha/year.

Measurement of forage yield by cutting and drying herbage is basically simple but there may be poor agreement between individual spaced plants and plots in the ranking of selections (Lazenby and Rogers 1964, Foster 1973). Harvesting method, grazing management and grass variety have a large impact on sward structure and composition. Barre et al. (2006) found that shorter leaves, produced as a result of environmental conditions or genetic selection, reduced intake rate in dairy cows. Mechanisation of forage plot harvesting in the 1970 s and 1980 s enabled more direct selection for plot yield and persistency, which had a major impact on selection progress. This was also helped by improvements in field data capture using portable computers and the application of NIRS to herbage analysis. Recent developments

Fig. 3 Forage plot harvesters equipped with a sampling device and a Near-Infrared Reflectance Spectrometer for 'online' dry matter and quality determination (Photo U. Feuerstein)

have also made it possible to use NIR with fresh herbage directly on a forage plot harvester (Figure 3; Feuerstein and Paul 2008, Paul et al. 2008).

6.1.2 Nutritional Quality – Digestibility

A key element in the economic and environmental sustainability of livestock systems is to minimise input costs through increased forage use. The nutritional composition of forage requires the same attention as other livestock feeds. Dry matter digestibility (DMD) is an overall measure of the nutritional value of herbage for ruminants that can be applied across a wide range of forage species (Casler 2000). It reflects the accessibility of plant cell wall polysaccharides to degrading enzymes in the rumen originating from rumen microorganisms or from within plant cells themselves (Kingston-Smith et al. 2008). Increasing DMD improves ruminant outputs by increasing rates of digestion, which raises voluntary intake. On average an increase of 1 unit of DMD improves animal output by 5% and allows farmers to reduce the amount of expensive high energy supplementary feeds needed to maintain economic levels of production. Ryegrasses generally have a high digestibility compared to other forage grasses and perennial ryegrass is more digestible than Italian ryegrass due mainly to the production of fewer stems. QTL for digestibility have been identified on chromosomes 1, 2, 5, 6 and 7; the same linkage groups as QTL for WSC and fibre (NDF) content (Van Loo et al. 2003, Vandewalle et al. 2003). Although it is not clear how QTL for component traits of nutritive value relate to DMD QTL, it is likely that marker selections for a range of traits can improve forage quality.

6.1.3 Nutritional Quality – Water-Soluble Carbohydrates

Plant cell contents are the most digestible component of herbage consisting mainly of a water-soluble carbohydrate fraction, a protein fraction and a lipid fraction.

The main reserve carbohydrates in temperate forage grasses are sucrose, fructan (polyfructosyl sucrose) and, to a lesser extent, starch (polymeric glucose). Concentrations of WSC in fresh herbage can reach 20–40% under field conditions and there is good potential for genetic improvement (Humphreys 1989, Feuerstein pers.com.). Under artificial conditions, temperate grass leaves can be induced to contain as much as 90% WSC (Cairns 2003). Utilisable genetic variation in WSC content in forage grass has been recognised for some time (Humphreys 1989) and conventional plant breeding has resulted in a number of successful commercial high-sugar varieties (e.g. AberMagic). A major problem for ruminant agriculture is that as little as 30% of ingested nitrogen is retained in milk or meat with the remainder being excreted to the environment as urea or ammonia. This inefficiency arises from the conversion of plant to microbial protein during rumen fermentation (Kingston-Smith et al. 2008) which depends on the availability of carbohydrates in the rumen during protein degradation. Higher sugar ryegrasses provide more available energy for rumen fermentation with significant improvements in ruminant productivity and reduced levels of excreted nitrogen (Miller et al. 2001). The value of silage as conserved winter feed also depends on readily available sugars for good fermentation. A minimum of 3.7% sugar is required in fresh grass for good-quality silage without the use of additives. WSC QTL in perennial ryegrass have been identified on chromosomes 1, 2, 5, 6 and 7 (Turner et al. 2006, see also Figure 10). Specific genes associated with fructan metabolism have also been mapped (Wei et al. 2000, Lidgett et al. 2002, Johnson et al. 2003).

6.1.4 Nutritional Quality – Protein

Protein levels are linked to grass growth stage and are affected by soil nutrition. With high nitrogen fertiliser inputs, levels can peak in early spring at above 30%. However N uptake is also influenced by potash and sulphur levels and soil pH. Van Loo et al. (2003) found QTL for NUE traits in ryegrass on chromosomes 1, 2, 4 and 5. Humphreys (unpublished data) identified crude protein QTL on chromosomes 2 and 3 and Vandewalle et al. (2003) reported a crude protein QTL on chromosome 4. Marker selection for positive NUE produced increased dry matter yield, reduced nitrogen content and increased sugar content (Van Loo et al. 2003).

6.1.5 Nutritional Quality – Lipids

Omega-3 polyunsaturated fatty acid (linolenic acid) and conjugated linolenic acid (CLA) in the human diet prevent the occurrence of cancer and heart diseases. Beside linseed oil cake, grass and grassland products are important vegetal sources of linolenic acid. A high content of polyunsaturated fatty acids (PUFA) in forage leads to higher meat and milk PUFA and CLA contents although impacts are reduced by plant lipases causing biohydrogenation in the rumen. Ryegrass species and varieties vary in their fatty acid content (Dewhurst et al. 2001) and provide breeding opportunities. Mixing grass with clover in forage leads to a higher Omega-3 content in

the milk than pure grass forage (Dewhurst et al. 2003) and there are indications that PPO (polyphenol oxidase) activity may also be important (Lee et al. 2004).

6.1.6 Abiotic Stress

As the tiller density of sown species declines, swards become invaded by weeds which reduce seasonal yield and nutritional value. Ryegrasses vary in their persistency depending on their innate perenniality and their ability to cope with abiotic (freezing temperatures, prolonged snow cover, low light intensities during winter, heat, drought, high light intensities leading to oxidative damage, anoxia resulting from ice encasement, flooding and slurry application, and high concentrations of aluminium, salt, manganese or heavy metals in the soil) and biotic (pests and diseases) stresses. Drought is a complex phenomenon and in temperate grasses the basic requirement for sward survival must be balanced against the need for rapid regrowth whenever water becomes available following drought. Many Mediterranean grasses such as *F. glaucescens* become 'quiescent' early with the onset of drought. Drought resistance results from a combination of traits that are not all independent from one another and a balance must be sought depending on the severity of the stress (Humphreys et al. 2006). Breeding targets include floral phenology, which determines indirectly the amount of growth devoted to roots and the density of vegetative tillers; root depth and water status; leaf production and extension; and regulation of transpiration (Humphreys and Humphreys 2005).

In conditions of water and nutrient stress endophyte infection may provide some benefits (Latch et al. 1985, Ravel et al. 1997). Endophyte infection levels were observed to increase more than twofold in perennial ryegrass pastures exposed to 2 years of severe drought (Reed et al. 2000). However, abiotic stress tolerance effects appear to be specific to particular perennial ryegrass–endophyte combinations (Cheplick and Cho 2003, Hesse et al. 2004) and are difficult to predict.

Winter survival depends on a range of adaptations including tolerance of freezing temperatures, carbohydrate storage and disease resistance under snow cover. Development of winter hardiness requires exposure of plants to low non-freezing temperatures, typically 0–10°C, and shortened photoperiod. Many physiological and biochemical changes occur during cold acclimation (Humphreys et al. 2006), including reduced growth and tissue water content, altered cell pH, protoplasm viscosity and photosynthetic pigments, reduced ATP levels, transient increases in ABA, changes in membrane lipids, accumulation of compatible solutes (e.g. proline, betaine, polyols and soluble sugars) and accumulation of antioxidants. Temperate grasses store fructans, a soluble polymer capable of rapid polymerisation and depolymerisation. The partitioning of solutes is important because survival from freezing depends on survival of tiller apices, particularly the lateral buds rather than mature leaf tissue (Eagles et al. 1993). The rate and extent of de-hardening is also critical, and temperature fluctuations can be very damaging (Gay and Eagles 1991). If climate change results in warmer winters, a strategy for winter survival based more on response to photoperiod rather than low temperature might gain importance as a target trait.

6.1.7 Diseases

Rusts (*Puccinia* species) can be particularly damaging pathogens of ryegrass since they reduce herbage sugar content and digestibility as well as yield (Potter 1987). It is estimated that crown rust infection level of just 10% can result in a 1.4% reduction in digestibility and is therefore very costly. In more extreme cases crown rust will cause long-term damage to swards and accelerate the need for reseeding. In recent years, there has been significant progress in mapping crown rust resistance genes in ryegrass (Muylle et al. 2005, Schejbel et al. 2007, Sim et al. 2007, Studer et al. 2007). Most of the described crown rust resistance in *Lolium* appeared to be quantitatively inherited, and QTL explaining a significant part of the phenotypic variation have been mapped on LG1, 2, 3, 4, 5 and 7. However, the pathogen *Puccinia coronata* is highly variable, and each of 30 perennial ryegrass genotypes investigated by Schubiger et al. (2007) had a different pattern of response to 106 European crown rust isolates. Therefore, the apparent quantitative nature of the inheritance of crown rust resistance is likely the result of the combined action of several qualitative resistance genes on a heterogeneous pathogen population. Another important leaf disease is caused by *Drechslera* ssp. (Lewis 1992) and genetic variation for resistance appears to exist although improvement through breeding is difficult.

Ergot (*Claviceps purpurea*) infects a wide range of grasses when cool wet conditions occur during flowering. The disease can spread rapidly from inflorescence to inflorescence. Dark ergot bodies are produced in infected florets, which contaminate seed lots and can be very difficult to remove.

Snow cover provides a favourable environment for the growth of other fungal pathogens. Resistance to these pathogens can be increased by selecting for natural resistance although the importance of various fungal species varies considerably with site (Boller et al. 1994). Grasses with larger tillers tend to be less susceptible to snow moulds, so forage ryegrasses are often less susceptible than turfgrass varieties, and tetraploids tend to be less susceptible than diploids. Turfgrasses are generally subject to a broader range of pathogens compared to faster growing forage types (American Phytopathological Society 1983).

6.1.8 Pests

Damage to grass swards by insects and other invertebrate pests can be serious, particularly with highly digestible grasses. Chemical control is neither practical nor environmentally desirable and consequently emphasis is placed on improving or maintaining plant resistance and tolerance. Perennial ryegrass may also gain benefits from a symbiotic association with the fungal endophyte *Neotyphodium lolii* (Latch et al. 1985, van Zijll de Jong et al. 2008) and infected plants are found in varying proportions within old pastures. Endophytes occur mainly in the intercellular spaces of the basal meristems and leaf sheaths of grasses, but during reproductive growth hyphae can grow up developing stems into the young inflorescences and ultimately infect mature seed (Philipson and Christey 1986). Unlike

the related endophyte species *Epichloe typhina* that causes choke in perennial ryegrass, *Neotyphodium lolii* lacks the ability to reproduce sexually and dissemination occurs through infected seed from the host plant which also provides nutrients and some protection for the fungus.

Specific host plant–endophyte combinations produce a range of alkaloid metabolites whose concentrations have been shown to be partly under ryegrass host genetic control (Easton et al. 2002). Lolitrem B is harmful to livestock at high levels and causes ryegrass staggers which produce tremors and loss of coordination particularly under heat stress. However peramine and lolitrem B provide protection against Argentine stem weevil (*Listronotus bonariensis* Kuschel), while ergovaline protects against African black beetle (*Heteronychus arator* F.). Endophyte strains that lack the alkaloids toxic to grazing livestock but retain those reducing pest damage have been introduced into perennial ryegrass varieties (Easton 2007, van Zijll de Jong et al. 2008).

6.1.9 Seed Yield

Tradeoffs between vegetative and reproductive growth continually challenge forage plant breeders. Improvement of vegetative traits like leafiness, tillering capacity and persistency of forage species may lower their ability to produce seeds. Components of seed yield that contribute to an increased utilisation of reproductive potential, such as seed set and seed retention may be an efficient way of increasing seed yield without adverse effects on the vegetative production. QTL mapping, identification of markers and candidate genes associated with seed yield components, and the use of comparative genomics with cereal species have revealed several key components which may facilitate development of markers for marker-assisted breeding for the improvement of seed yield. The importance of seed yield in breeding programmes is discussed further in Section 9.

6.2 Breeding Goals in Italian Ryegrass

In general the main objectives of Italian ryegrass breeding are similar to those for perennial ryegrass and include high dry matter yield (DMY), high nutritive value and high seed yield. Improving early spring and autumn DMY is particularly important as it allows seasonal production to be extended. For short-term silage species, such as Italian ryegrass, a high first cut yield is vital. Because of the more frequent sowing of Italian ryegrass for short-term use, including green manure, a low seed price based on a high seed yield is more important than for perennial ryegrass. Persistency and cold tolerance are less important criteria but are still taken into account. Breeding for resistance to diseases such as crown rust, stem rust, brown rust, leaf spot and bacterial wilt is equally important as it is for perennial ryegrass. Crown rust (*Puccinia graminis*) is the major disease but *L. multiflorum* is also highly susceptible to bacterial wilt (*Xanthomonas translucens* pv. *graminis*) (Rechsteiner

et al. 2006). Concerning nutritive value, digestibility and water-soluble carbohydrate content are the traits most focussed on. Redfearm et al. (2002) evaluated differences in cumulative forage yield, yield distribution and nutritive value among six cultivars of annual ryegrass. No significant differences for cumulative forage yield were observed. However, significant differences for forage quality indicate potential to meet specific requirements in terms of forage need and season of use (Andrés et al. 2005).

Measured traits in the official variety tests of most European countries for *L. multiflorum* generally include yield (usually dry matter yield, total and first cut), heading date, ground cover, earliness, persistence and disease resistance. Except for UK and Ireland, crown rust resistance is always reported on and, depending on the country, resistance to other diseases are also listed (including other rusts, virus, *Xanthomonas*, *Fusarium*, *Drechslera* or more generally 'leaf' diseases). Several lists report resistance to lodging, winter hardiness or persistence under snow. Heading in the first year is sometimes reported as a negative point for *L. multiflorum.* Digestibility is reported only on the lists of Switzerland and UK.

The aftermath digestibility (% of dry matter) of Italian ryegrass varieties on the UK Recommended List (2005/2006) ranges from 61.9 to 64.0%, compared to 62.2 to 67.1% for hybrid ryegrass and 66.3 to 71.1% for perennial ryegrasses. The mean annual DMD of Italian ryegrass varieties in Belgian trials was similar to meadow fescue, 6% higher than timothy and 3% lower than perennial ryegrass at high and low nitrogen application levels (Baert et al. 1999). Tetraploid Italian ryegrass varieties listed in Switzerland (2009/10 list) had generally higher digestibility than diploid varieties. This was also true within the early and late groups of *L. perenne.*

Digestibility is variable through the year, being the highest at the first cut (year after sowing) and dropping in the following cuts when more flowering stems appear. Reheul and Ghesquiere (1994) investigated which cut or combination of cuts best reflects the digestibility of ryegrasses over a whole year (four to five cuts). For Italian ryegrass, they concluded that if only one cut had to be chosen it should be cut 4 and that for a combination of two cuts, it should be cuts 2 and 4. In the UK official trials, digestibility determination takes place only at the second conservation cut (aftermath DMD). This might be responsible for the low digestibility percentages reported for Italian ryegrass varieties on this list. Aavola (2007) studied the forage quality improvement of Italian and perennial ryegrass by applying different mineral fertilisation and cutting frequencies. It was concluded that in a breeding program aiming at feeding value improvement, it is necessary to measure digestibility and intake, regardless of management conditions.

Water-soluble carbohydrate percentage is not included in any of the official variety lists for ryegrass. Published data for Italian ryegrass is scarce (McGrath 1988, Marais et al. 2003) but Carlier (1974) found a lower WSC percentage in Italian ryegrass compared to perennial ryegrass varieties. *L multiflorum* families, synthetics and varieties in trials at ILVO (Ghesquiere et al. 2008) had a mean %WSC (in dry matter harvested over five cuts) of 15.25 in 2004 and 11.65 in 2005. These were lower than the %WSC obtained for *L. perenne* entries in the same trials which varied around 16% in both years. Tetraploid *L. multiflorum* entries had a 1.9 and

0.9% higher WSC content compared to diploids in 2004 and 2005 respectively. As expected from previous work in *L. perenne*, Vandewalle (2007) found for *L. multiflorum* high positive correlations between WSC and DMD, as well as relatively high negative correlations between CP and WSC.

With regard to crude protein (CP) concentration, Redfearm et al. (2002) found significant differences among harvests (2 cuts × 2 growing seasons) for six *L. multiflorum* varieties with a general decrease from 260 to 120 g CP/kg as the growing season progressed. In Japan, nitrate poisoning in ruminants caused by the accumulation of nitrate in forage crops can be a serious concern. Inoue et al. (2000) studied varietal difference in nitrate nitrogen content in Italian ryegrass. Content ranged from 33 to 1,237 ppm, was lower in late maturing varieties and lower in varieties with fewer stems. Harada et al. (2003) developed a seedling test for large-scale selection for the breeding of low nitrate populations. In a pot experiment the nitrate concentration of the adult plants of the third recurrent generation was 60% of the value of the original population.

Dewhurst et al. (2001) studied the influence of species, cutting date and cutting interval on the fatty acid composition of grasses. *L. multiflorum* and *Lolium hybridum* had higher levels of total fatty acids and alpha-linolenic acid in the early and late season compared to perennial ryegrass.

With regard to diseases, bacterial wilt (*Xanthomonas translucens pv. Graminis (Xtg)*) is an increasingly significant problem in Italian ryegrass. First observed in the early 1970 s (Egli et al. 1975), bacterial wilt is now prevalent in the grasslands of Europe, the USA and Australasia causing substantial yield losses and reduced persistency (Channon and Hissett 1984). Host plant infection occurs through stomata and epidermal wounding so that mowing facilitates infection and spread. Several measures of disease control have been considered, including disinfection of contaminated mowing equipment and inoculation with epiphytic bacteria to induce disease resistance. However, these methods had limited success in the field (Schmidt 1988a, 1988b) and breeding for resistance is an attractive target. Italian ryegrass varieties with considerable levels of disease resistance were produced initially (Lehmann et al. 2000) although highly susceptible plants are still observed after many cycles of recurrent selection (Michel 2001). Studer et al. (2006) found a single major QTL on linkage group (LG) 4 explaining 43–84% of the total phenotypic variance for resistance.

The most widespread grass viruses belong to the barley yellow dwarf virus complex (Latch 1977). In the ryegrasses, infection usually reduces root growth, although shoot growth can be normal or even be increased by infection (Catherall and Parry 1987). Ryegrass mosaic virus is a problem in Italian and hybrid ryegrasses. It is spread by the eriophyid mite *Abacarus hystrix,* rather than through mowing (Heard and Chapman 1986). No true resistance to ryegrass mosaic virus has been found within Italian ryegrass, but high levels of resistance to systemic infection as well as resistance to virus spread and multiplication have been found in perennial ryegrass (Salehuzzaman and Wilkins 1984). Genes conferring resistance to systemic infection in ryegrass are additive in action and thus hybrid ryegrasses can be partially resistant.

Resistance to the herbicide glyphosate appeared around 1996 for *L. rigidum* followed by many different species. Resistance in *L. multiflorum* was reported first in 2003 and mechanisms have been investigated by Perez-Jones et al. (2007).

6.3 Breeding Goals in Westerwolths Ryegrass

Following the shift in breeding programmes of Westerwolths ryegrass from main crop to catch crop performance, there has been little change in specific goals for Westerwolths ryegrass breeding in the last 20 years. In many regions Westerwolths ryegrass is sown in the autumn as winter pasture and therefore good growth during this relatively cold season is important. Under these conditions crown rust (*Puccinia coronata*) is the main problem and nearly all Westerwolths ryegrass breeding programmes concentrate on breeding for rust resistance. Prine (1990) described a mass selection procedure in spaced-plant nurseries of about 20,000–30,000 single plants using spreader rows of the rust susceptible perennial ryegrass variety 'Manhattan'. Soon after detection of the first rust spots on the Westerwolths plants roguing was carried out to remove severely rusted plants. The healthy survivors formed a breeding population assumed to contain accumulated rust resistance genes.

Under mild conditions, overwintering catch crops of Westerwolths ryegrass permit the multiplication of various vectors of diseases of other important crops. In France selection has been undertaken to reduce this. For example, a number of different programs have aimed to breed for nematode resistance. Resistance to the cereal cyst nematode (*Heterodera avenae*) has been achieved by the breeding company Carneau in France with the Westerwolths variety 'Cannibal' (Rivoal and Bourdon 2005).

There are only a few breeding programmes that target quality aspects such as the improvement of ensilability in Germany and general forage quality in South Africa.

7 Breeding Methods and Specific Techniques

7.1 General Methods with Specific Reference to Perennial Ryegrass

Domestication of forage crops is relatively recent with targeted breeding to improve landraces and wild species ecotypes commencing only early in the twentieth century. The collection and exploitation of natural variation from ecotypes and landraces have been important in improving temperate grasses since then. As breeders attempt to incorporate a greater range of improvements into their varieties, the possibilities of finding suitable combinations of traits within ecotypes become limited and new gene pools must be created by intercrossing diverse genetic resources (Figure 4). Wilkins (1991) found large amounts of genetic variation for yield in the progeny of early × late perennial ryegrass accessions and distant crossing has been

Fig. 4 Series of pair crosses between individuals of Italian ryegrass with the aim of creating a new gene pool as starting population for selection. A strong air current through bags ensures mutual pollination of each pair of plants and isolation from other crosses (Photo J. Baert)

used very effectively in the improvement of ryegrasses for yield, nutritive value and persistency.

Because most temperate grasses are perennial out-breeders, some form of recurrent selection is usually employed prior to creating synthetic varieties based on a variable number of parents. This is an effective way of concentrating desirable genes in an out-breeding gene pool. A generalised example of a ryegrass breeding scheme is shown in Figure 5.

Casler and Brummer (2008) discussed the advantages of using among-and-within family (AWF) selection to make the most efficient use of additive genetic variance within half-sib or full-sib families. They argued that AWF selection is better than progeny-test selection particularly when (i) there is high heritability on an individual-plant basis (relative to heritability on a family-mean basis), (ii) within family selection intensity $\geq$ among-family selection intensity. They indicated that these conditions are frequently achieved for half-sib mating systems due to the greater partitioning of additive genetic variance within families, but may also be favoured in a full-sib mating system.

Recurrent phenotypic selection is often used in ryegrass breeding with limited progeny testing (Wilkins and Humphreys 2003). Direct selection for plot performance may take place only once every 10 years or more, and selection intensity is low. Some breeders incorporate full-sib or half-sib family selection into breeding cycles in order to increase direct selection for plot performance. In perennial ryegrass Wilkins and Humphreys (2003) described how four generations of combined phenotypic and half-sib family selection over 12 years was successful in simultaneously improving dry matter yield and water-soluble carbohydrate content in perennial ryegrass. In Ireland, half-sib and full-sib family selection has been used to produce tetraploid ryegrass varieties with improved yields (Connolly

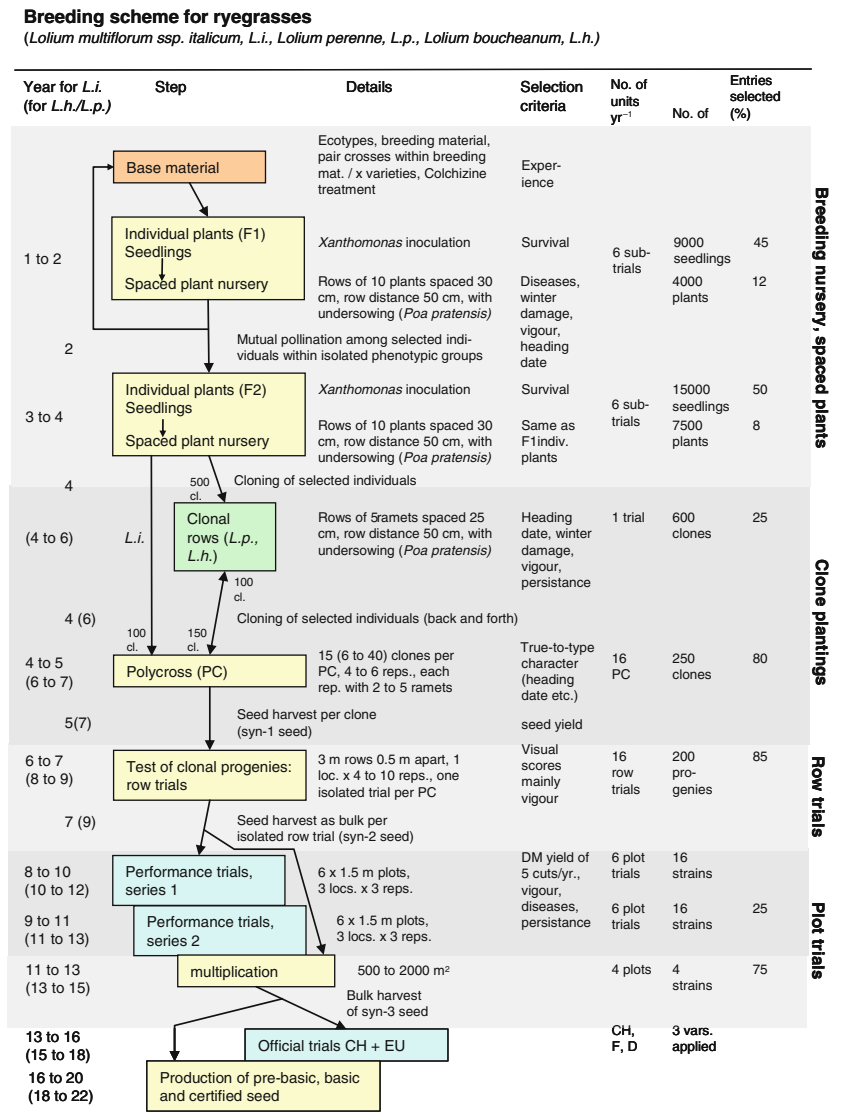
Breeding scheme for ryegrasses
(Lolium multiflorum ssp. italicum, L.i., Lolium perenne, L.p., Lolium boucheanum, L.h.)
Year for L.i. (for L.h./L.p.)
Step
Details
Selection criteria
No. of units yr⁻¹
No. of
Entries selected (%)
Base material
Ecotypes, breeding material, pair crosses within breeding mat. / x varieties, Colchizine treatment
Experience
Individual plants (F1) Seedlings
Spaced plant nursery
1 to 2
Xanthomonas inoculation
Survival
6 sub-trials
9000 seedlings
45
Rows of 10 plants spaced 30 cm, row distance 50 cm, with undersowing (Poa pratensis)
Diseases, winter damage, vigour, heading date
4000 plants
12
2
Mutual pollination among selected individuals within isolated phenotypic groups
Individual plants (F2) Seedlings
Spaced plant nursery
3 to 4
Xanthomonas inoculation
Survival
6 sub-trials
15000 seedlings
50
Rows of 10 plants spaced 30 cm, row distance 50 cm, with undersowing (Poa pratensis)
Same as F1indiv. plants
7500 plants
8
4
500 cl.
Cloning of selected individuals
Breeding nursery, spaced plants
Clonal rows (L.p., L.h.)
(4 to 6)
L.i.
Rows of 5ramets spaced 25 cm, row distance 50 cm, with undersowing (Poa pratensis)
Heading date, winter damage, vigour, persistance
1 trial
600 clones
25
100 cl.
4 (6)
Cloning of selected individuals (back and forth)
100 cl.
150 cl.
Polycross (PC)
4 to 5 (6 to 7)
15 (6 to 40) clones per PC, 4 to 6 reps., each rep. with 2 to 5 ramets
True-to-type character (heading date etc.)
16 PC
250 clones
80
Clone plantings
5(7)
Seed harvest per clone (syn-1 seed)
seed yield
Test of clonal progenies: row trials
6 to 7 (8 to 9)
3 m rows 0.5 m apart, 1 loc. x 4 to 10 reps., one isolated trial per PC
Visual scores mainly vigour
16 row trials
200 progenies
85
Row trials
7 (9)
Seed harvest as bulk per isolated row trial (syn-2 seed)
Performance trials, series 1
8 to 10 (10 to 12)
6 x 1.5 m plots, 3 locs. x 3 reps.
DM yield of 5 cuts/yr., vigour, diseases, persistance
6 plot trials
16 strains
Performance trials, series 2
9 to 11 (11 to 13)
6 x 1.5 m plots, 3 locs. x 3 reps.
6 plot trials
16 strains
25
Plot trials
multiplication
11 to 13 (13 to 15)
500 to 2000 m²
4 plots
4 strains
75
Bulk harvest of syn-3 seed
13 to 16 (15 to 18)
Official trials CH + EU
CH, F, D
3 vars. applied
16 to 20 (18 to 22)
Production of pre-basic, basic and certified seed

Fig. 5 A generalised ryegrass breeding scheme used at ART Reckenholz, Switzerland (B. Boller pers. com.)

2001). Combined phenotypic and half-sib family selection has also been used to improve perennial ryegrass in France (Ravel and Charmet 1996). Wilkins and Humphreys (2003) also pointed out that the main limitation of recurrent family selection is the number of family plots that can be evaluated at each generation. Broad-sense heritabilities for yield, digestibility and ground cover often range from

30 to 70%, and at least four replicate plots are needed to give optimal selection responses.

During recurrent selection it is vital to avoid excessive inbreeding and genetic drift which may reduce growth rate, yield and seed set (Utz and Oettler 1978). Ryegrasses have two main self-incompatibility loci (S and Z) and varieties should incorporate a sufficiently high number of S and Z alleles to maintain heterozygosity at both loci for good seed set. During marker-assisted selection (MAS) it is useful to know the relative location of target genes to incompatibility loci so that loss of fertility can be avoided. Before Plant Variety Rights regulations were implemented with UPOV guidelines for Distinctness, Uniformity and Stability (DUS), grass varieties were often based on large numbers (20–30) of unrelated parents. These were sometimes substituted by better genotypes as they emerged and hence varieties tended to 'evolve' with time. Subsequently DUS restrictions resulted in reducing the number of parents in a synthetic to between 4 and 10, often from the same half-sib or even full-sib family. The optimal number of clones depends on their general combining ability and potential for inbreeding depression. More component genotypes ensure less inbreeding but also reduce selection intensity with an increasing risk of genetic drift during seed production. According to Posselt (2000) the optimum number of parents for a synthetic variety lies in the range of 5–15. With increasing inbreeding depression the optimum number increases. In both diploid and tetraploid ryegrasses it appears that inbreeding depression related to herbage yield is not high but seed yield may be reduced significantly in varieties based on only 2 parents (Ghesquiere and Baert 2007, Baert et al. 2008).

High selection intensity restricts the genetic base of varieties and reduces the amount of additive variation segregating within varieties which may limit genetic flexibility. Genetic markers can be used to assess genetic diversity in breeding populations and indicate how gene pools may benefit from the introduction of new variation. As the range of selection criteria facing breeders increases, it is inevitable that forms of multitrait selection will be used. Incorporating genetic markers into selection indices can help to increase selection efficiency, avoid undesirable correlated responses and predict interactions with environmental factors.

Much of the progress achieved to date in grass breeding has relied on the accumulation of additive gene effects in synthetic varieties with little exploitation of the non-additive genetic variation associated with heterosis. Selection of polycross parents for high molecular marker diversity is a possibility to exploit heterosis and avoid inbreeding depression in the construction of forage grass synthetics (Kölliker et al. 2005). Boller et al. (2008) found that selection for high molecular diversity can improve agronomic performance of grass synthetics. However, the difference in diversity must be large to obtain significant results.

In seed-based varieties, rapid exploitation of heterosis depends on some form of pollination control, e.g. male sterility; the manipulation of self-incompatibility systems; or by the controlled use of apomixis. Cytoplasmic male sterility (cms) has been introduced into perennial ryegrass from meadow fescue (Connolly and Wright-Turner 1984), but the production of F_1 hybrid seed is expensive and may not be

economic. Gaue and Baudis (2007) describe using a cms system induced by chemical treatment. Using the two-locus incompatibility system to produce predominately F_1 hybrid seed by intercrossing two partly inbred families at the final generation of seed production is another possibility (Posselt 1993). In practice it is difficult to compensate for higher seed prices of hybrids with sufficient improvements in performance. An alternative approach is provided by tetraploid hybrid ryegrasses which are based on a pair of tetraploid Italian and perennial ryegrass parents. Preferential pairing between chromosomes of the parent species preserves heterozygosity and provides adequate stability over generations of seed production without pollination control (Jones and Humphreys 1993).

So far, introgression of individual genes by backcrossing has played a limited role in developing new forage varieties compared to recurrent selection. Introgression was used successfully to introduce resistance to blind seed disease (*Gloeotinia temulenta*) into S24 perennial ryegrass (Wright and Faulkner 1982). Despite a close genetic relationship, *L. perenne* and *F. pratensis* demonstrate physiological and metabolic differences for many agronomically important traits (Thomas and Humphreys 1991). Fescue traits of value include improved drought tolerance, colour, nutritive value (including reduced protein degradation and fatty acid content and composition), mineral uptake, cold tolerance, durable disease resistance and general persistency. Targeted introgression of traits includes transfer of *F. pratensis* chromosome segments to *L. perenne* that improve winter hardiness, crown rust resistance and retention of green foliar colour. Drought resistance has been transferred from hexaploid *F. arundinacea* and tetraploid *F. glaucescens* to three different sites on chromosome 3 of *L. multiflorum* (Humphreys et al. 2006). Ryegrass families with enhanced drought resistance and heat tolerance have also been developed from hybrids involving North African *F. mairei* (a close relative of *F. glaucescens*).

7.1.1 Developments in Phenotyping

To make significant progress in plant breeding it is necessary to make large numbers of measurements with a high degree of accuracy. Although yield is relatively easy to assess, it is not an entirely straightforward trait. There may be poor agreement between individual spaced plants and plots in genotype ranking for annual dry matter yield (DMY) (Lazenby and Rogers 1964, Foster 1973). Index selection of perennial ryegrass spaced plants in France improved several traits but not yield (Ravel et al. 1995). Selection within perennial ryegrass for spaced-plant DMY in the UK actually reduced plot yield (Hayward and Vivero 1984). This problem was somewhat alleviated by the development of plot harvesters which enable efficient direct selection for plot yield and persistency. With a widening range of ‘sustainability’ traits and novel ‘quality’ components for forage and biofermentation/biofuel, the availability of new techniques for more accurate, rapid and non-invasive phenotyping is becoming increasingly important. Traditional field phenotyping is still useful, e.g. for disease monitoring, while hydroponics provides highly controlled conditions for phenotype analysis of ‘sustainability’ traits. However, to match

progress in the development of DNA-based markers for genotyping, it is necessary to exploit advanced metabolic phenotyping techniques.

Already techniques such as Fourier Transform Infrared (FT-IR) and Near Infra-red Reflectance Spectrometry (NIRS) are being used routinely to close the genotype–phenotype gap. NIRS is a fast and low cost spectroscopic method, based on measuring absorbance or reflectance of NIR light (wavelength range 700–2500 nm). Since NIRS was introduced by Norris et al. in 1976 as a new method of forage analysis, its cost effectiveness has supported its widespread use in plant breeding and variety testing to evaluate forage quality (Feuerstein and Paul 2008). NIRS is now used routinely to determine digestibility, protein and carbohydrate contents in forage breeding and to assess the clover content of mixed swards. Calibration models are also emerging for secondary compounds such as lignin and ergovaline. Due to limitations of instrumentation, NIRS has been used mainly under stationary laboratory conditions. However in the late 1990 s high speed diode array sensors for near infrared with high temperature stability and mechanical robustness were developed which now permit fresh forage analysis even on harvesters under field conditions (Feuerstein and Paul 2008).

Breeding perennial ryegrass for amenity purposes (Thorogood 2003) is very similar to breeding for forage use. Phenotypic recurrent selection is commonly used together with some half-sib progeny testing. Individual genotypes from polycrosses can supply sufficient seed to test for turf quality in small scale plot performance trials.

7.2 Specific Breeding Methods in Italian Ryegrass

As in perennial ryegrass, the most common method of Italian ryegrass improvement is based on a recurrent selection strategy. It consists of repeated cycles of selection; each cycle involves the evaluation of a population of plants, identification of a subset of superior genotypes and intercrossing of the selected plants. An example of a scheme used at ILVO (Belgium) is given in Figure 6.

Depending on the selection aim the starting population is more or less restricted and directed selection for specific criteria is performed. Usually, the initial phenotypic selection takes place on individual spaced plants. Cloning of superior plants for further evaluation is used for Italian ryegrass as for perennial ryegrass, however the risk of losing interesting genotypes is high due to low persistency. Interesting genotypes are grouped for seed production (usually as a polycross with all possible combinations). Progeny tests per component may be performed and subsequently varieties constructed and evaluated. The cross might also be evaluated as a whole and used directly for breeders seed because the maintenance of Italian ryegrass genotype clones is not easy. As with perennial ryegrass selected genotypes are grouped according to their morphological characters. Flowering time may also be considered for grouping genotypes although compared to perennial ryegrass its range is very small.

Even though breeding cycles for Italian ryegrass are shorter than for perennial ryegrass, evaluation of plot performance takes place only after 4–6 years (without

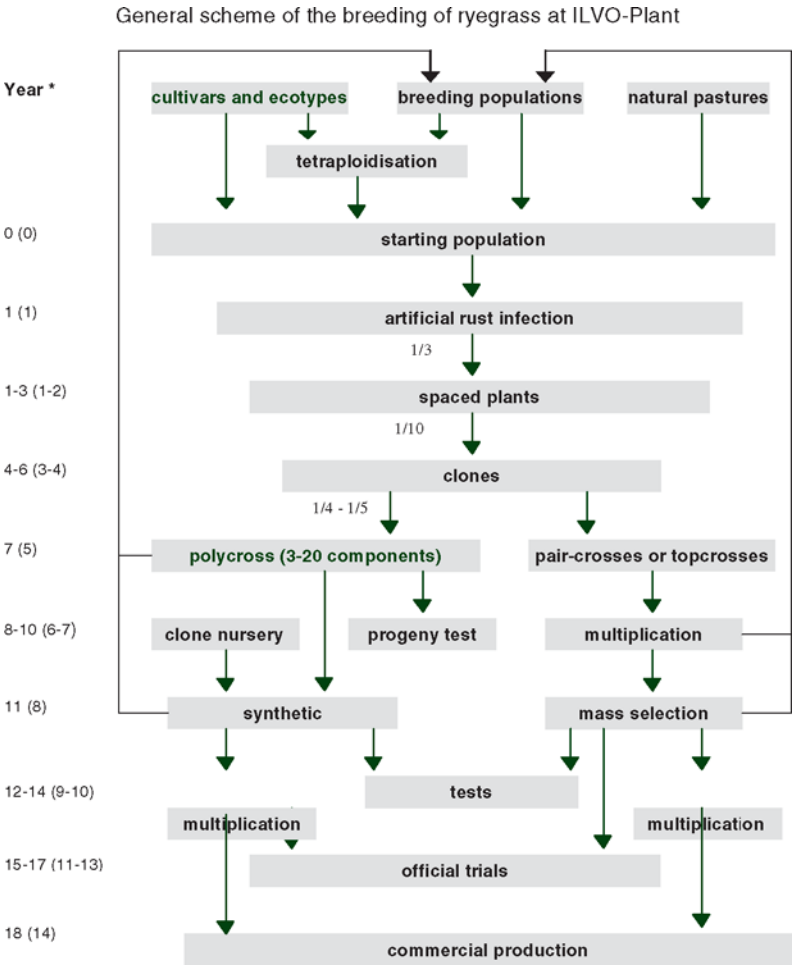

Fig. 6 Illustration of a recurrent polycross selection method used for ryegrasses at ILVO, Belgium. *The numbers of years are approximate and might vary; years between brackets indicate the Italian ryegrass scheme, while the years without brackets indicate the perennial ryegrass scheme. Based on Frandsen and Frandsen (1948), and adaptations from Reheul and Baert (personal communication) and from Van Bockstaele and Baert (2004)

or with clone evaluation) resulting in a low selection intensity (Van Bockstaele and Baert 2004). Most breeding programs now include quality traits such as digestibility, crude protein and water-soluble carbohydrates. However these determinations often take place on harvested material from progeny plots. Many breeding programs now also include routine selection for crown rust and *Xanthomonas* resistance under artificial infection (Reheul and Ghesquiere 1996), and selection for growth under low nitrogen conditions.

7.3 Specific Breeding Methods in Westerwolths Ryegrass

The most common breeding methods in Westerwolths ryegrass are based on mass selection and progeny selection approaches which breeders adapt to their own breeding schemes.

7.3.1 Mass Selection

In many cases mass selection is not very efficient because of uncontrolled gene flow during flowering, but forage plants have an advantage in that selection can be carried out before flowering. Before flowering selected plants can be removed and planted together in isolation or can remain in the breeding nursery if undesirable plants are destroyed before flowering. For mass selection it is a precondition that genetic variability exists for a specific trait in a given population. It is often effective only for highly heritable traits such as heading date or some disease resistance traits such as rust resistance. An example given by Prine (1990) is illustrated in Figure 7.

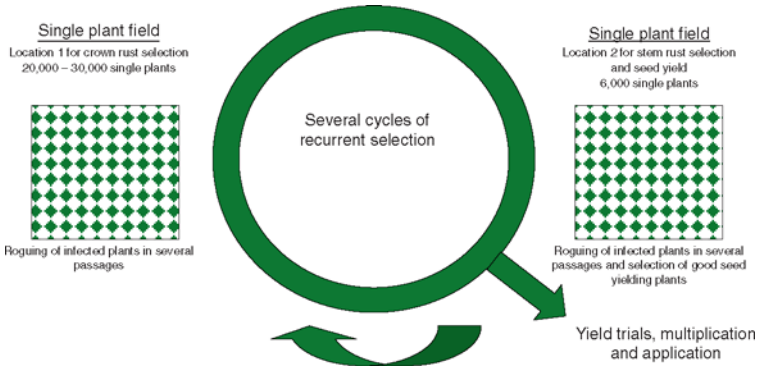
Single plant field
Location 1 for crown rust selection
20,000 – 30,000 single plants
Roguing of infected plants in several passages
Several cycles of recurrent selection
Single plant field
Location 2 for stem rust selection and seed yield
6,000 single plants
Roguing of infected plants in several passages and selection of good seed yielding plants
Yield trials, multiplication and application

Fig. 7 Recurrent mass selection in Westerwolths ryegrass

El-Shamarka (1988) provides another example where mass selection proved to be adequate in improving 'ensilability' (estimated as an index of dry matter content, buffering capacity and WSC content in freshly harvested forage). In a population of 97 progenies he observed sufficient variability for 'ensilability' to allow a divergent selection. Dry matter content and buffering capacity showed the highest heritability ($h^2 = 0.74$ and 0.61 respectively). However in the absence of much genetic variation for WSC content the ensilability index was only moderately heritable ($h^2 = 0.49$).

7.3.2 Indirect Selection

In many cases it is very laborious, costly or even impossible to analyse a trait and breeders look for possibilities to carry out indirect selection. El-Shamarka (1988) detected a very close positive correlation between dry matter content and ensilability. After summer sowing dry matter yield showed a close relationship with the

proportion of heads in autumn. Because heading date had a much higher heritability than ensilability, indirect selection for early heading under short day conditions was much more efficient than direct selection for improved ensilability with a lower heritability. This approach is illustrated in Figures 8 and 9.

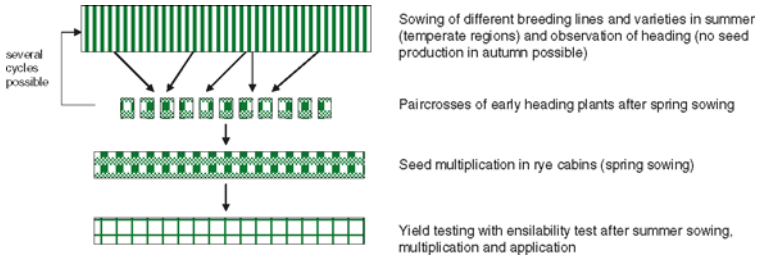
several cycles possible
Sowing of different breeding lines and varieties in summer (temperate regions) and observation of heading (no seed production in autumn possible)
Paircrosses of early heading plants after spring sowing
Seed multiplication in rye cabins (spring sowing)
Yield testing with ensilability test after summer sowing, multiplication and application

Fig. 8 Indirect selection for ensilability under catch crop conditions in Westerwolths ryegrass

Fig. 9 Isolation of pair crosses among selected individuals of ryegrass in rye cabins (Photo U. Feuerstein)

8 Integration of New Biotechnologies into Breeding Programmes

Modern crop improvement can use a range of genetic, biochemical, phenotypic, computational and modelling approaches to assist in targeted molecular-based breeding. The links between genotype and phenotype are becoming clearer with genomic approaches used to determine the genetic control of complex traits. New phenomics research provides unique data sets associated with plant morphology, chemical composition, metabolism and physiology, alongside genotyping and crop performance data. These support predictive modelling of future varieties and help

to identify key traits for breeding programmes. The development of DNA markers allows precise targeting of genes for marker-assisted selection.

Perennial, outbreeding grasses offer challenges that are distinct from many other crops. However, they also provide opportunities to gain greater insight into basic biological phenomena such as metabolic control, the evolution of perenniality and leaf senescence using genomic resources from model species such as rice and *Brachypodium*. Information of generic value can be obtained from ryegrass which has advantages compared to other crop plant species particularly with regard to the integration of linkage disequilibrium analysis with QTL mapping and allele mining in diverse germplasm. Grasses within the *Lolium–Festuca* species complex contain an exceptionally wide range of variation, including adaptations to contrasting temperatures, day length, rainfall and soils. Interspecific and intergeneric recombination rates in *Lolium–Festuca* are the highest amongst monocot crops, allowing introgression to be exploited as a highly efficient research and breeding tool, and assisting in exchanges of genomic information across all monocot species (Humphreys et al. 2003).

8.1 The Use of DNA Markers

Genetic maps together with QTL and linkage disequilibrium analysis can locate genes associated with important agronomic traits and hence facilitate marker-assisted selection (MAS) and introgression in crop breeding programmes. *Lolium* genetic maps are well aligned with those of other grass species, including rice, barley and wheat (Jones et al. 2002. Microsynteny is becoming well established with gene orthologues being identified across species (Armstead et al. 2004, Jensen et al. 2005).

High throughput DNA markers for genotyping and MAS are increasing the scale and precision of plant breeding (Yadav et al. 2003). MAS is particularly useful when traits are difficult or expensive to evaluate, including late expression in growth cycles; or when several genes need to be pyramided including the need to improve several traits simultaneously; or when selection of recessive alleles is important. It also assists in the introduction of new alleles into breeding populations to help enable recurrent selection maintain progress over many generations.

Although linkage mapping and QTL analysis in forage grasses initially lagged behind that in cereals, considerable progress has been made in recent years particularly within the *Lolium–Festuca* species complex. Most groups working with *Festuca* and *Lolium* now have good maps for QTL analysis underpinning MAS (e.g. Van Loo et al. 2003, Armstead et al. 2004, Yamada et al. 2004).

8.2 DNA Markers in *Lolium perenne*

Initial work to identify QTL in ryegrass was carried out in the early 1990 s based on a partial linkage map including isozyme loci (Hayward et al. 1994). More detailed maps and QTL studies developed during the following decade. Yamada et al. (2004)

described QTL for morphological traits together with information on physiological traits such as winter hardiness and WSC content. If the expression of a trait is highly dependent on the environment, then phenotyping must be carried out in different environments in order to gain a full picture of the genetic control of the trait in relation to QTL analysis (Turner et al. 2006, Figure 10 Turner pers com.).

QTL validation has been carried out using marker selection with some success in *L. perenne* (Humphreys and Turner, 2003, van Loo et al. 2003). Differences in phenotype may be small if selections are based on a single QTL and if segregation occurs at other regions of the genome controlling the trait. Selections based on indices combining several QTL have produced greater effects (Dolstra et al. 2007). When random markers are used to characterise QTL regions, it is advisable to identify fairly large regions of chromosome to be sure of transferring the trait of interest. However SNP markers in candidate genes underlying QTL are becoming available (Skøt et al. 2007) which allow selections to be based on smaller regions of the chromosome and hence minimise the risk of selecting undesirable linked traits. The ILGI reference map for perennial ryegrass (Jones et al. 2002) was aligned with the Triticeae consensus map and hence with rice which helps to identify functional gene markers in ryegrass (Andersen and Lübberstedt 2003, Armstead et al. 2008, Forster et al. 2008).

Limitations of QTL analysis to characterise traits of interest in outbreeding species such as *Lolium* and *Festuca* can arise from the narrow genetic base of mapping families in comparison to the total range of variation available. Different regions of the genome may be identified as controlling a given trait in different mapping families of the same species, even if, in a given cross, one QTL appears to explain a large part of the variation. This is clearly demonstrated with heading date in *Lolium*, where QTL have been identified on linkage groups 2, 4, 6 and 7 in various studies (Yamada et al. 2004, Armstead et al. 2004, Jensen et al. 2005). Other approaches, such as introgression mapping and recent developments in association mapping, may be better at providing a means to access the full range of variation available (Skøt et al. 2007).

8.3 DNA Markers in *Lolium multiflorum*

Although many DNA markers were produced initially in perennial ryegrass (e.g. Muylle et al. 2003), markers developed in Italian ryegrass are now also available (Hirata et al. 2006, Miura et al. 2007, Inoue and Cai 2004). Similarly, most genetic maps were initially developed in perennial ryegrass, although there are a few based on hybrids between Italian and perennial ryegrass or on pure Italian ryegrass (Table 6).

QTL analyses carried out using Italian ryegrass, including interspecific *L. multiflorum* × *L. perenne* hybrid populations, are listed in Table 7. Hayward et al. (1994) identified QTL for phenological traits and dry matter yield. Using the same three generation interspecific (Lp × Lm) ryegrass population, Curley et al. (2005) and Jo et al. (2008) detected QTL for resistance to grey leaf spot (*Magnaporthe grisea*), leaf

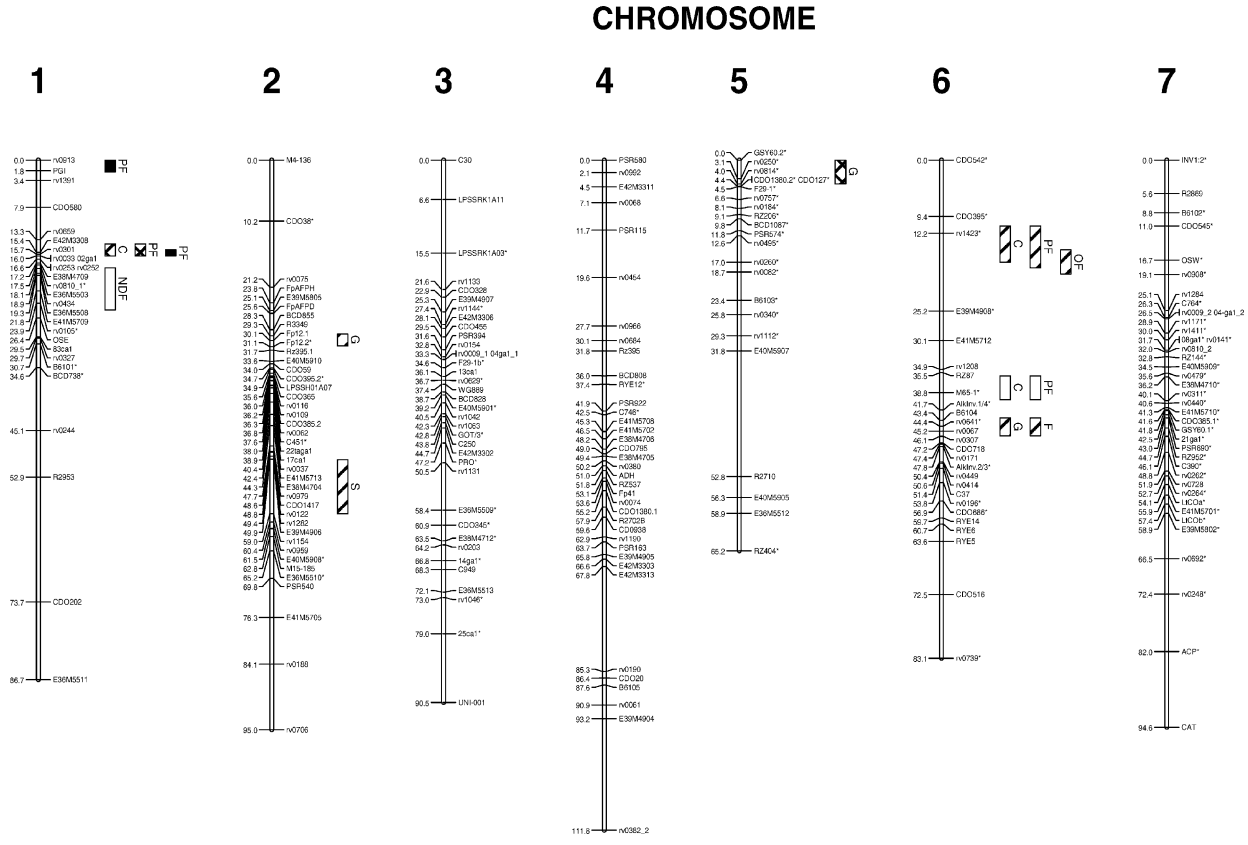

Fig. 10 Forage quality QTL consistent across years. Chromosome designations conform to the Triticeae numbering. Markers with a distorted segregation ratio ($P < 0.05$) are indicated $*$. *QTL bars* represent the 1 LOD fall-interval. *Open bars* are leaf QTL in the spring, *hatched bars* are leaf QTL in the autumn, *cross-hatched bars* are tiller base QTL in the spring and *filled bars* are tiller base QTL in the autumn. Total WSC, C; polymeric fructan, PF; oligomeric fructan DP>3, OF; sucrose, S; glucose, G; fructose, F; neutral detergent fibre, NDF

Table 6 Summary of published genetic maps for L. multiflorum (Lm). DH: doubled haploid, Lp: *L. perenne*

Species	Type of segregating population	Marker types	Map length	Authors
Interspecific Lp and Lm	DH (Lp) x F1 (Lp × Lm) 89 plants	RAPD, RFLP, Isozyme	750 cM 692 cM	Hayward et al. 1994, 1998
Lm	F1-population 227 plants	AFLP, STS, SSR	1372 cM	Muylle et al. 2003
Lm	F1-population 82 plants	RFLP, AFLP, TAS**	1244 cM	Inoue et al. 2004
Interspecific Lp and Lm	F1 × F1 (Lp x Lm) 91 plants	RAPD, RFLP, AFLP, SSR, Isozyme	537 cM (♀) 712 cM (♂)	Warnke et al. 2004
	F1 × F1 (Lp x Lm) 152 plants	RAPD, RFLP, AFLP, SSR, Isozyme	664 cM	Sim et al. 2005
Lm	F1-population (CMS) 60 plants	RFLP, AFLP, SSR	888 cM (♀) 796 cM (♂)	Hirata et al. 2006
		RFLP, AFLP, SSR, EST derived CAPS	906 cM (♀) 800 cM (♂)	Miura et al. 2007
Lm	F1-population 306 plants	AFLP, SSR	804 cM	Studer et al. 2006
Lm	4 F1-populations 100 plants each	AFLP, SSR, STS	689 to 925 cM	Vandewalle 2007

** TAS = Telomeric repeat-associated sequence markers

spot (*Bipolaris sorokiniana*) and stem rust (*Puccinia graminis*). Studies using pure Italian ryegrass populations have focussed on heading date and lodging resistance (Inoue et al. 2004), bacterial wilt resistance (Studer et al. 2006), crown rust resistance (Muylle et al. 2005, Studer et al. 2007) or yield and quality traits (Vandewalle 2007). Little consistency across populations was observed by Vandewalle (2007) for yield and quality traits. QTL analyses for traits including photoperiodic control of flowering time and crown rust resistance have also been presented (e.g. Warnke et al. 2004). Bulked segregant analysis was used in Italian ryegrasses by Gao et al. (2002) to establish linkage of AFLP markers to *lhd* 1, a recessive heterochronic gene, and by Hackauf and Lellbach (2008) to map a major dominant gene *(LmPc)* conferring resistance to crown rust**.**

So far, QTL analysis in *L. multiflorum* has not led to successful marker-assisted selection. The attempt made by Vandewalle (2007) to select for identified QTL markers did not deliver expected gains in yield or quality. Studies in *L. multiflorum* are now focussing on identifying candidate genes for MAS breeding and investigating the molecular basis of traits such as bacterial wilt resistance (Rechsteiner et al. 2006).

Table 7 Summary of QTL analysis in *L. multiflorum* (Lm). Lp: *L. perenne,* BSA: bulk segregant analysis, SMR: single-marker regression, SIM: simple interval mapping, KW: Kruskal-Wallis, MQM: multiple QTL mapping, CIM: composite interval mapping)

Species	Population	Analysis	Trait(s)	Authors
Interspecific Lp and Lm	1-way pseudotestcross DH (Lp) × F1 (Lp × Lm) 89 plants	Retrospective method, Oneway ANOVA	No. inflorescence, ear emergence, hay cut yield, ear in first year	Hayward et al. 1994
Lm	2-way pseudotestcross F1-population 227 plants	BSA, SIM, MQM	Crown rust resistance	Muylle et al. 2003, 2005
Lm	2-way pseudotestcross F1-population 82 plants	SIM, CIM	Heading date, lodging resistance	Inoue et al. 2004
Interspecific Lp and Lm	'3-generation population'	SIM, KW, MQM	Gray leaf spot resistance	Curley et al. 2005
	F1 × F1 (Lp x Lm) 152 plants	SIM, KW, MQM	Multiple disease resistance (leaf spot, stem rust)	Jo et al. 2008
Lm	2-way pseudotestcross	SIM, MQM	Bacterial wilt resistance	Studer et al. 2006
	F1-population 306 plants	MQM	Crown rust resistance	Studer et al. 2007
Lm	8 unrelated parents 4 F1-populations 100 plants each	SIM, KW	Yield and quality traits	Vandewalle 2007

8.4 Genetic Transformation in Ryegrasses

Although natural ecotypic variation continues to provide the basic raw material for most forage breeding programmes, developments in cell culture and transformation techniques provide opportunities to access new sources of variation beyond that currently available using conventional techniques. However use of transformation technology is limited by (i) a lack of precise information on the physiological, morphological and biochemical consequences of individual gene action as well as gene identification and cloning; (ii) 'Trade-offs' in whole plant performance that may be alleviated by targeting genes to specific tissues or by using appropriate genetic backgrounds; (iii) problems of stability in the expression and inheritance of transgenes; (iv) a need for specific management practices and variety evaluation systems that recognise the value of novel material (v) statutory regulation linked to public awareness of benefits and risks; (vi) environmental concerns due to a high

probability of gene flow from transgenic plants in out-crossing species, evidence of long-range pollen flow and the existence of feral/wild populations of the same species. In ryegrasses there are no genetic barriers and often no geographical barriers to introgression of transgenes into naturally occurring wild populations, resulting in potentially high risks of gene transfer associated with any release of GM varieties and a corresponding need for risk assessment.

Increasingly *Agrobacterium* mediated transformation is being used in ryegrasses (Sato and Takamizo 2006). Primary target traits include forage quality, disease and pest resistance, tolerance to abiotic stresses, and the manipulation of growth and development. Wu et al. (2005) obtained salt-tolerant perennial ryegrass by transformation with a rice vacuolar membrane Na+/H+ antiporter gene *(OsNHX1)*. Drought tolerance was improved in perennial ryegrass through transformation and over-expression of the *CBF3/DREB1A* gene (Han et al. 2008) which forms part of the abscisic acid-independent stress-response pathway in *Arabidopsis*. Gadegaard et al. (2008) developed perennial ryegrasses with a 3-fold increase in fructan content based on the expression of *sucrose:sucrose 1-fructosyltransferase* and *fructan:fructan 6G-fructosyltransferase* genes from onion. Transgenic Italian ryegrass plants have been produced which express traits such as altered fructan accumulation (Ye et al. 2001), increased resistance to crown rust (Takahashi and Fujimori 2005) and down-regulation of main pollen allergens (Petrovska et al. 2004).

9 Seed Production

9.1 Commercial Relevance

Although forage grasses are bred primarily for agronomic characteristics such as yield, persistency and nutritive value, the ability to produce reasonable seed yields is essential to ensure that new varieties are taken up in practice. The commercial relevance of ryegrass seed production is considerable. About 50% of the grass seed used in Europe is perennial ryegrass. The total certified seed production area of perennial ryegrass in the EU-27 was 89,476 ha in 2006/07 (36% of total EU-27 grass seed production). Abberton et al. (2008) considered the impact of climatic conditions on grass species utilisation across Europe. In Norway perennial ryegrass use is confined to areas with the least winter stress and only 200 t/year of seed is used in 2-year swards. This compares with 1,000 t/year of Timothy (*Phleum pratense*) and 400 t/year of meadow fescue (*F. pratensis*). In contrast, 75% of the seed marketed annually in the UK is perennial ryegrass (10,000 t/yr) and 13% is *L. multiflorum*. In Poland, about 1,000 t/year of perennial ryegrass seed is used currently for re-seeding meadows and pastures as part of complex seed mixtures comprising between 15 and 50% *L. perenne* (information provided by Germinal Holdings (UK), Szelejewo Plant Breeding, Poland, and the Norwegian Crops Research Institute; EU FPV project SAGES http://www.iger.bbsrc.ac.uk/SAGES2/sages2.html).

In 2006/07 the total area of Italian ryegrass seed crops (including Westerwolths) accepted for certification in the EU-27 was 35,928 ha (14.5% of the total EU-27 grass seed production area). Within Europe grass seeds are produced mainly in Denmark (35% in 2006) followed by Germany (13.6%), the Netherlands and France (+/−10% each). About 40,000 t of Italian and Westerwolths ryegrass seed is produced annually mainly in Germany, the Czech Republic and France.

Diversity in seed size within and between genetic types (diploid and tetraploid) and cultivars is large. Tetraploid varieties have about 40% larger seeds than diploid varieties. The 1000-seed weight for a single cultivar may have a range of 1–4 g. On average, the 1000-seed weight of Italian ryegrass varieties is higher than that of perennial ryegrass and usually ranges from 1.5 to 3 g for diploids and from 3 to 5 g for tetraploids.

9.2 Breeding for Seed Productivity

As with most temperate outbreeding forage grasses, ryegrasses go through several stages in the initiation and induction of growing points during flower development. Initially, there is a vegetative or juvenile stage when shoot meristems produce leaves and tillers. During autumn and early winter, there is a mature meristem response to low temperatures (vernalisation) and/or day length (short day) which induces a tiller to become reproductive. This fundamental process of tiller development underlies a wide range of variation in plant maturity and persistence.

Seed yields in perennial ryegrass are variable and many of the characteristics associated with high seed yields are often negatively correlated with herbage yield and persistence. Improvements in digestibility have been achieved partly by reducing the proportion of tillers becoming reproductive (Wilkins and Humphreys 2003) which again may reduce seed yields. Despite considerable genetic variation for seed yield and its components (Elgersma 1990, Rognli 2008), breeding for improved seed yield has received little attention. The typical reproductive tiller in perennial ryegrass is a simple spike of 18–25 spikelets. High yielding ryegrass seed crops typically produce 2,000–2,200 kg seed ha^{-1} from a seed head density of 1,800–2,500 spikes m^{-2} (Rolston et al. 1997). There was little variation in the number of spikelets per spike among a range of cultivars (Elgersma 1990) and seed yield improvement is more likely to be attained through increasing the number of seeds per spikelet and improved floret site utilisation. Marshall and Wilkins (2003) successfully carried out two generations of recurrent phenotypic selection for seed yield per plant under controlled pollination. The increased seed yield was attributed to a higher proportion of ovules forming seeds (% seed set), greater seed number per tiller and more reproductive tillers per plant. Studer et al. (2008) found that seed yield per panicle produced the highest effect on total seed yield. Of particular interest were two QTL on linkage group (LG) 1 and LG 2, explaining 41 and 18%, respectively, of the observed phenotypic variation for the trait seed yield per panicle. Both QTL are co-located with two major QTL for total seed yield per plant, possibly representing the S and Z loci of the gametophytic self-incompatibility (SI)

system of perennial ryegrass. The diversity of SI alleles in mapping parents and the degree of heterozygosity at SI loci in the full sib progeny determine the interference of self-incompatibility with seed production. The genetic characterisation of SI alleles in breeding populations using functional markers for S and Z loci (Andersen and Lübberstedt 2003) is of major interest in order to avoid SI-mediated seed yield losses. This is of particular importance for second cycle breeding or synthetics based on few components strongly selected for traits that are linked to S and Z, thereby narrowing the diversity of SI alleles.

The production of organic forage seed to meet seed certification standards is a significant challenge. Conventional systems of grass seed production use inorganic nitrogen applied at specific stages of crop development and appropriate herbicides to produce high quality seed, reduce weed content and minimise seed cleaning costs. The development of organically acceptable methods of weed control and techniques of applying nitrogen at precise stages of crop development are critical for successful organic grass seed production (Marshall and Humphreys 2002). In tetraploid and diploid perennial ryegrass, seed yield in organic production is decreased by about 25% compared to conventional production.

9.3 Seed Production in *Lolium multiflorum*

Seed production in Italian ryegrass differs from perennial and Westerwolths ryegrass in that one or two fodder cuts can be taken in the spring before the seed harvest cut. In this way, weeds are eliminated without the need for chemical application after sowing. European Italian ryegrass seed production currently takes place mainly in Germany, Czech Republic and France. Other world-wide production sites of importance are New Zealand and Oregon, USA. The European average production for 2006/07 was 1,113 kg ha^{-1}. Seeding rate for seed production crops ranges from 15 to 25 kg/ha for diploids and from 20 to 30 kg/ha for tetraploids. Nitrogen application depends on the soil type and the number of fodder cuts taken in addition to seed harvest. Usually, 90–130 kg N/ha is allocated to fodder cuts and 60–100 kg N/ha to the seed cuts (Kunelius et al. 2004).

9.4 Seed Production in Westerwolths Ryegrass

Seed production in Westerwolths ryegrass is relatively simple. Sowing of the seed crop takes place in early spring without a cover crop and the seed harvest is taken in the same year. In regions without extreme winters, a late autumn sowing is also possible. Seed crop sowing rate is normally between 12 and 16 kg/ha for diploids and 16 and 22 kg/ha for tetraploids. Nitrogen application depends on the expected seed yield. Low yielding varieties should get about 40 kg/ha after establishment and 20 kg/ha at time of ear emergence whereas high yielding varieties should get more at the second application date (e.g. 60 kg/ha). In most cases direct combining will be used for harvest, but swathing and picking up later by a combine is also

possible. Only one seed harvest can be taken from a crop. The seed harvest potential of Westerwolths ryegrass is higher than for Italian or perennial ryegrasses but there are large differences between varieties.

Seed production of Westerwolths ryegrass is possible in most parts of the world but large differences in seed yield can be expected. Many varieties are produced in the Willamette River Valley of Western Oregon, USA. In Canada the range of seed yields is between 213 kg/ha and 1,210 kg/ha. Earlier varieties yielded much better than later varieties ($r = -0.85$). There is also a clear relationship between tiller density and seed yield ($r = 0.81$ and 0.98 in Canada and Germany respectively). High seed yielding varieties tend to have a relatively low regrowth after harvest ($r = -0.50$). In warm regions Westerwolths ryegrass is often used as a reseeding crop. Evers and Nelson (2000) analysed the reseeding capacity of different Westerwolths ryegrasses after different dates of grazing termination in Texas (USA). In this area it was possible to graze most varieties until late of April and to get still enough heads for reseeding. Natural reseeding of Westerwolths ryegrass is also known in New Zealand, Australia and South America.

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Fescues

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1 Introduction

Fescues are very diverse grasses which are important components of natural, permanent, and intensively managed grasslands, lawns, and turfs, and are used for conservation purposes. Fescue (*Festuca* spp.) species can be divided into two groups; the broad-leaved fescues meadow fescue (*F. pratensis* Huds.) and tall fescue (*F. arundinacea* Schreb.), and the fine-leaved fescues. Fine fescues are grouped into *Festuca rubra* (red fescue) and *Festuca ovina* (sheep fescue) complexes or aggregates. The *F. ovina* group includes the hard fescue, sheep fescue, and blue fescues. The genus *Festuca* L. is distributed mostly in the temperate zones of both hemispheres; most abundant all around the Northern Hemisphere (Jenkin 1959).

Meadow fescue (*F. pratensis* Huds.) is a forage grass of high quality and yield potential considered native to Europe and Eurasia (Hultén and Fries 1986). In Europe it is distributed throughout the climatic regions of oceanic northwest Europe and the transitional oceanic/continental zone of central Europe (Borrill et al. 1976). Meadow fescue constitutes a significant component of species-rich permanent pastures and hay fields in alpine regions and in eastern Europe. It was probably introduced to Scandinavia from Europe and West Asia, and has since become naturalized, and it was also introduced to North America, Japan, Australia, and New Zealand. In North America the acreage of meadow fescue has been rather insignificant relative to the more drought tolerant tall fescue, however, Casler et al. (1998) concluded that meadow fescue may be more useful than tall fescue in intensive grazing management systems. Meadow fescue is also more prevalent at higher altitudes than tall fescue.

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Tall fescue (*F. arundinacea* Schreb.) is an important cool-season perennial forage grass species throughout the temperate regions of the world. It was introduced in the USA in the 1800s, but was not planted widely until the middle of the twentieth century. It is one of the most popular pasture grasses and cultivated on about 15 million ha in the USA (Buckner et al. 1979). Superior growth, adaptability to a wide range of soils and climates, responsiveness to N fertilization, high tolerance to grazing, and availability of forage over much of the year are key reasons for its popularity over other forage grasses. Tall fescue demonstrates good shade tolerance and remains green all-year-round under irrigated conditions in cooler climates. Although tall fescue originates from Europe and northern Africa it is less widely accepted in the European market. Under intensive cultivation perennial ryegrass (*Lolium perenne* L.) is in general preferred because of its better grazing persistence and palatability. However, due to changing climatic conditions and the availability of improved cultivars the acceptance of tall fescue for forage purposes is increasing. Apart from being used as forage, its uses extend to lawns, turf, and conservation purposes (Sleper and Buckner 1995). This chapter mainly focuses on the forage-type tall fescue.

Fine fescues are a group of cool season perennial grasses that are commercially and agronomically valued for forage, turf, landscape, and ornamental purposes. Fine fescues have very fine and narrow leaves that minimize the water loss through transpiration and give them good drought tolerance. Some of the important fine fescues include red fescue, Chewings fescue, sheep fescue, hard fescue, and blue fescue among many other species. Fine fescues tolerate shade, drought, low pH (5.5–6.5), and low soil fertility (Beard 1973, Hanson et al. 1969), and require little to no additional inputs of fertilizer or supplemental irrigation (Ruemmele et al. 1995). These grasses are predominantly used in the turf industry owing to their low maintenance and other agronomic features. In their native habitats, that include cool season regions of Europe, Asia, and North America, fine fescues occur on permanent grasslands used for forage.

2 Origin and Systematics

Festuca is a very large cosmopolitan genus comprising about 450 species whose taxonomic relationships with other closely related genera has been much debated (Clayton and Renvoize 1986). Many species of the *Festuca–Lolium* complex have very similar morphological features and both spontaneous and induced intergeneric hybrids can be found. Stebbins (1956) proposed that *Lolium* should belong to *Festuca*, and Darbyshire (1993) reclassified the broad-leaved species of the genus *Festuca* into *Lolium*. Studies using chloroplast DNA restriction sites and sequence-based phylogenies have supported the placement of the genus *Lolium* together with *Festuca* in the tribe Poeae of the grass family Poaceae (Soreng et al. 1990; Mathews et al. 2000). AFLP and SSR markers were used to conduct genetic diversity and phylogenetic analysis of grass species (Saha et al. 2004, Mian et al. 2005). Microsatellite analysis of *Festuca* and *Lolium* species indicated that tall

fescue and meadow fescue were grouped together while red fescue (*F. rubra*) was grouped with *Dactylis glomerata* (orchardgrass). The relationship of red fescue with orchardgrass was also supported by another study (Charmet et al. 1997). Tetraploid tall fescue (*F. glaucescens*) was more closely related to hexaploid tall fescue (*F. arundinacea*) than to meadow fescue. The similarity matrix based on DNA sequences depicts tall fescue as having a very high sequence similarity with all other *Festuca–Lolium* species (Mian et al. 2005). Of the grass species outside the *Festuca–Lolium* complex, orchardgrass had the most (96.6%) and rice the least (88.3%) similarity to tall fescue. According to the rate of nucleotide substitution in chloroplast DNA (cpDNA) spacers, broad-leaved and fine-leaved fescues diverged some 9 Mya (Charmet et al. 1997, Fjellheim et al. 2006). A dendogram of the *Festuca/Lolium* complex obtained from cpDNA, ribosomal DNA (rDNA), and ITSs analyses showed the same differentiation of the three major groups: (i) fine-leaved fescues; (ii) broad-leaved fescues; and (iii) ryegrasses (Charmet et al. 1997).

Meadow fescue (*F. pratensis* Huds.) is diploid ($2n = 2x = 14$) and obligate outbreeding with a two-locus (S and Z) gametophytic self-incompatibility system (Lundqvist 1962). The variation present in cpDNA of meadow fescue is very restricted, only three haplotypes demonstrating distinct geographical structuring were found among meadow fescue genotypes representing the present distribution area of the species (Fjellheim et al. 2006). The putative origin of these haplotypes points toward migration of meadow fescue from alpine regions of Iberia, the Alps, and Caucasus after the last glaciation. Lower cpDNA variation in meadow fescue than in polyploid fescues, e.g., tall fescue that derives from it, indicates that meadow fescue went through a bottleneck during or after the last glaciation. Today, *F. pratensis* is widespread in the open agricultural landscape but appears otherwise confined to naturally open habitats such as river banks and wetlands, and its populations may have been decimated when dense forests dominated in the previous interglacial (Fjellheim et al. 2006). Contrary to meadow fescue, studies of cpDNA variation in *L. perenne* have detected a large amount of variation (Balfourier et al. 2000, McGrath et al. 2007), and this is puzzling in view of the close relationship between these two species. The geographic structuring of cpDNA variation in *L. perenne* points to migration of this species associated with the expansion of agriculture from the Fertile Crescent after the last glaciation (Balfourier et al. 2000). There is no evidence for such a migration of meadow fescue. A tetraploid form (*F. pratensis* var. *apennina* (De Not.) Hack.) is present at altitudes above 1100 m in the Alps, the Carpathian Mountains, and the Apennini Mountains (Tyler 1988) and natural triploid hybrids between sympatric diploid and tetraploid meadow fescue can be found. Earlier studies suggested that var. *apennina* probably is allo- rather than autotetraploid in origin containing two closely related genomes one of which is *F. pratensis* (Lewis 1977). However, var. *apennina* appears to be cross-fertile with colchicine induced autotetraploid *F. pratensis* (Boller pers. comm., Sugiyama and Gotoh 1987).

Tall fescue (*F. arundinacea* var. *genuina* Schreb.) is an allohexaploid ($2n = 6x = 42$) species with genomic constitution PPG1G1G2G2 and disomic inheritance. The

P genome is derived from the diploid progenitor meadow fescue while the G1G2 genomes are from tetraploid tall fescue mostly referred to as *F. glaucescens* (*F. arundinacea* var. *glaucescens* Boiss; 2n = 4x = 28) (Xu et al. 1991). Tall fescue is native to Europe and North Africa. The European and North African ecotypes have very distinct characteristics and ploidy levels, and are adapted to very different environmental conditions indicating separate evolution north and south of the Mediterranean Sea. The northern European ecotypes were mainly introduced into North America. Though tall fescue gained popularity only in the dryer parts of its native Europe and less so in areas of intensive forage production, it became one of the most popular forage grass species in North America. Increased interest for tall fescue in Europe arises from expectations related to climatic changes such as increased frequency of dry periods during summer.

Many agronomically important fine fescues belong to the subgenus *Festuca*. Within this subgenus, fine fescues are grouped into *Festuca rubra* (red fescue) and *Festuca ovina* (sheep fescue) complexes or aggregates. Classifying species as either of the aggregate is generally not difficult and is based on leaf blade anatomy, leaf sheath morphology, root fluorescence, or cytology (Huff and Palazzo 1998), but it is difficult to characterize species within each of the two aggregates. Studies have shown a wide range of chromosome numbers ranging from diploid to decaploid sets in fine fescues.

3 Varietal Groups

Although there is a certain amount of variation in heading time among meadow fescue cultivars, there are no specific varietal groups like early and late maturity groups for this species. An exception from this is the extremely late heading Japanese cultivar 'Hokkai9', which was bred from the Finnish cultivar 'Tammisto'. The late heading appears to be the result of a long-day response when grown at lower latitudes than those of it's origin like in Japan (Takai et al. 2004). Late heading is also generally associated with lower seed yields. Meadow fescue has its main advantage as a companion species. It is very rarely sown in monoculture; most often it is sown in mixtures with other grasses and clovers. A typical seed mixture for Scandinavia would be timothy (*Phleum pratense* L.), meadow fescue, and red clover (*Trifolium pratense* L.).

There are two major tall fescue germplasm pools, Continental and Mediterranean. Continental germplasm originates from central and northern Europe, is relatively winter-hardy and remains in active growth throughout the year in North America. Mediterranean germplasm which traces back to southern Europe, the Middle East, and North Africa is generally less winter-hardy, shows summer dormancy, and grows well at moderately low temperatures and/or short nights. Genetic barriers are usually seen in crosses between Mediterranean and Continental types even with uniform ploidy levels with meiotic irregularities within progenies (Hunt and Sleper 1981). Rhizomatous tall fescue is sometimes

considered a third germplasm pool. These are believed to originate from northwest Spain and Portugal but strongly rhizomatous populations are also found in other regions of Europe. Continental germplasms can also form rhizomes (Bouton et al. 1992), but the rhizomes are narrower and less prevalent than the rhizomatous germplasm. Rhizomatous germplasm is less winter-hardy. Hybrids between rhizomatous germplasms with either Continental or Mediterranean types can be highly sterile (Carlson and Hurst 1989). The 'Rhizomatous Tall Fescue' (RTF) cultivar 'Labarinth', released by Barenbrug USA in 2002, was developed by topcrossing a Portuguese rhizomatous tall fescue ecotype with American and European turf-type tall fescue selections (Singh et al. 2005). This cultivar produces almost normal levels of seed yield, although more variable than 'normal' tall fescue, and there are improved cultivars in the pipe-line with more stable seed yields (K. de Bruijn pers. comm.).

There are two distinct functional tall fescue groups, i.e., forage and turf types. Forage-type germplasms are characterized by coarse leaves, upright growth habit, and tall plants. 'Alta' and 'Kentucky-31' are the two oldest varieties. 'Kentucky-31' is still one of the most popular and widely grown tall fescue cultivars in the USA. In Europe breeding for soft-leaved types has set new benchmarks and extended the usage of tall fescue. The cultivar 'Barcel' was the first breakthrough, 'Bariane' and later 'Dulcia' and 'Belfine' followed. In soft-leaved types, the saw-like silicium teeth prevalent on leaf borders and ridges of coarse-leaved tall fescue are reduced to small rounded bulb-like structures (Figure 1). Turf types have finer dark green leaves, short growth stature, and dense tillers. Contrary to forage types, turf types can tolerate frequent and close mowing. Development of turf-type cultivars was mainly initiated in the 1970s. 'Rebel,' 'Olympic,' 'Houndog,' 'Falcon', and 'Adventure' were eventually released as turf-type cultivars in the USA.

Most of the agronomically important fine fescues are grouped either into the *Festuca rubra* or *Festuca ovina* complexes; as such they do not seem to constitute any varietal groups (see also Chapter 6). Based on the plant morphology, anthesis, and cytological analyses, the species within the *F. rubra* complex are distinguished from the species within the *F. ovina* complex (Schmit et al. 1974). Within the *F. rubra* aggregate, strong creeping red fescue (*F. rubra* ssp. *rubra* L.) and slender creeping red fescue (*F. rubra* ssp. *trichophylla*) belong to the rhizomatous groups due to the presence of rhizomes whereas chewings fescue (*F. nigrescens* Lam., syn. *F. rubra* ssp. *commutata* Gaudin) is a bunch type grass that can be easily separated from the creeping fescues based on the absence of extravaginal stems or rhizomes (Figure 2). Distinguishing the two creeping red fescues based on their morphological differences is more difficult. Red fescues have superior shade adaptation and generally have high shoot density, good uniformity, and fine textured medium to dark green leaves (Meyer and Funk 1989).

The *F. ovina* group includes the hard fescue, sheep fescue, and blue fescues. Identifying the species within this aggregate is most difficult and considerable confusion still exists among the European and American breeders over the classification of hard and sheep fescues (Ruemmele et al. 1995). In the USA, sheep fescue is described as having a bluish-gray leaf color whereas hard fescue leaf blades are

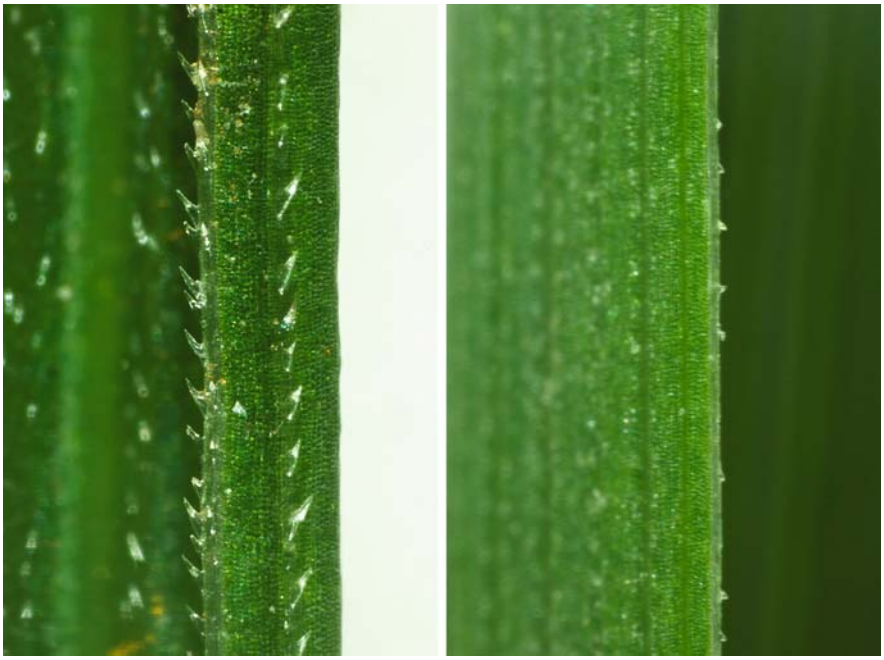

Fig. 1 Magnified view of leaf blades of coarse-leaved tall fescue cultivar 'Kora' (*left*) compared to soft-leaved cultivar 'Otaria' (*right*) (Photo G. Brändle)

considered to be green (Beard 1973, Turgeon 1991). The opposite criterion is followed in Europe. Hard fescues (Figure 3) have high shoot density, good shade adaptation, and can tolerate excess water better than sheep fescue (Ruemmele et al. 1995). Sheep fescues have tufted growth habit, and have stiffer leaves than hard fescues (Meyer and Funk 1989). Blue fescues are durable grasses with good disease resistance. These are low maintenance grasses when compared to other fine fescues and can tolerate very poor soils (Meyer and Funk 1989).

4 Genetic Resources and Utilization

A search in the ECPGR *Festuca* Database hosted by the Nordic Genetic Resource Center (http://www.nordgen.org/ecpgr/) returns 12,288 records distributed as follows among the different species: *F. pratensis* (6,076), *F. arundinacea* (2,255), *F. ovina* (327), *F. rubra* L. (2,292) of which 127 accessions are classified as *F. rubra* ssp. *rubra* and 146 as *F. rubra* ssp. *commutata* (accessed on June 16, 2009). These accessions are stored at a number of European gene banks and represent nearly all countries of Europe. About half of the *F. pratensis* records (3,414) are from Poland.

Fig. 2 Spaced plants of species of the *Festuca rubra* complex used in turf breeding: rhizomatous creeping red fescue (*top left*) and slender creeping red fescue (*top right*); bunch type chewings fescue (*bottom*) (Photos N. Roulund)

Fig. 3 Spaced plant of hard fescue in a breeding nursery at Rutgers, New Jersey (Photo S. Bonos)

4.1 Meadow Fescue

Local (ecotypes) and naturalized populations in pastures and meadows plus cultivars are important genetic resources of meadow fescue that has been studied and utilized quite extensively. Meadow fescue is a significant component of species-rich permanent pastures and meadows in harsher climates, and Kölliker et al. (1998) claim that the relative abundance of meadow fescue has been decreasing over the years maybe due to limited genetic variability.

Significant genetic diversity was found within and between six natural populations collected from long-term experiments (11–38 years) and three cultivars of meadow fescue using RAPD markers and agronomic traits (Kölliker et al. 1998). Intensive management, i.e., fertilization and frequent defoliation led to a reduction in genetic variability within the natural populations. Also, molecular diversity and variability in phenotypic traits were considerably lower within cultivars of meadow fescue compared to cultivars of *L. perenne* and *D. glomerata* (Kölliker et al. 1999).

The Germplasm Resources Information Network (GRIN) (http://www.ars-grin.gov/, accessed on June 21, 2009) holds 312 accessions of *F. pratensis* and 11 accessions of *F. pratensis* ssp. *apennina*. Almost all of these accessions originate from Europe and Asia and consist of natural collections and cultivars. Casler and van Santen (2000) screened 213 of the GRIN accessions, mainly from Europe, at two locations in USA for variation in forage yield, disease reaction, morphological traits, maturity, and survival. They found large variation among accessions in adaptation to different environments and several accessions that might be useful as turf-type germplasm.

Fjellheim et al. (2007) found larger phenotypic diversity for a number of traits, except disease resistance, within local meadow fescue populations from Norway

compared to Nordic cultivars. As an example; variation in heading date was 3.5 days among cultivars while it was 9 days among local populations. Similarly, Casler and van Santen (2001) found that the variation was larger within naturalized accessions than cultivars from Eurasia. In a comparison of ecotypes from 19 habitats in Switzerland with cultivars it was found that several ecotype populations were superior to cultivars in important phenotypic traits demonstrating the value of natural habitats as a reservoir of genetic resources for breeding, calling for an in situ conservation strategy for grasslands (Peter-Schmid et al. 2008a).

Contrary to this, molecular diversity (AFLP) analyses of 30 local Norwegian populations and 13 Nordic cultivars showed little variation between both local populations and cultivars, and the level of variation within cultivars was higher than within local populations (Fjellheim and Rognli 2005b). This indicates that at least the Norwegian meadow fescue germplasm has a narrow genetic basis. However, the genetic diversity within newly released Nordic cultivars was as large as that of old cultivars, indicating that breeding has not eroded genetic diversity over time in the Nordic region (Fjellheim and Rognli 2005a).

Based on molecular (AFLP) diversity it has been shown that the local populations in Norway can be structured into three groups, western, southern, and inland, probably reflecting different routes of introduction of the species into Norway. Geographic structuring into three sub-clusters was also found in a study of 12 Swiss ecotype populations using SSR markers (Peter-Schmid et al. 2008b). The Norwegian inland populations are closely related to the cultivars and have most probably been established as a result of migration from sown meadows (Fjellheim and Rognli 2005b). This is also the case for Baltic natural populations which belong to a different gene pool than the Norwegian populations and reflect dispersal from different glacial refugia after the last glaciations (Fjellheim et al. 2009). The group of Baltic cultivars appears not structured with 91% of the molecular variation within cultivars, indicating narrow breeding populations of this species in the Nordic-Baltic region. All cultivars in this region are most closely related to local populations from the Baltic pointing toward extensive exchange of breeding materials among the public breeding stations in this region (Fjellheim et al. 2009). The Swiss ecotype populations studied by Peter-Schmid et al. (2008b) were clearly separated from cultivars and indicate that the natural populations of meadow fescue in the Alps are much older than populations in northern Europe.

4.2 Tall Fescue

The National Genetic Resources Program of the United States Department of Agriculture maintains an online database of tall fescue germplasm in the Germplasm Resources Information Network (GRIN). A total of 899 accessions have been collected from different parts of the world (http://www.ars-grin.gov/cgi-bin/npgs/html/tax_stat.pl accessed on April 28, 2009). More than half of these accessions (494) were collected from 20 European countries; France is the major contributor (214) followed by Spain (115). The second most contribution comes

from Africa (169) of which 112 accessions were collected from Morocco. A total of 105 accessions were collected from the United States. Six Asian countries contributed 69 germplasms. Minor collections were also made from Australia, former Soviet Republics, and South America. In Europe there are less well documented collections but nature is still preserving a lot of interesting biodiversity. However, there is lacking literature about the distribution of tall fescue in south and eastern Europe, Asia, and South America. For better understanding of their evolution denser tall fescue ecotype collections are needed in order to understand the way ecotypes are scattered over Europe due to glacial movements, sea levels, etc.

4.3 Fine Fescues

Native and adapted regions of Europe, Asia, and USA serve as germplasm resources of fine fescues. Public and private plant breeders have collected numerous samples from various parts of the world. Fine fescues collected from coastal areas and mining sites could be a useful source of saline and heavy metal tolerant germplasm. A wide range of germplasm collections among various species of fine fescues is present at the The Germplasm Resource Information Network (GRIN). Interspecific hybridization between various species of fine fescues may create variability with desirable characters from each of the parental species.

Sampoux and Huyghe (2009) investigated a large French collection of fine fescue accessions from a wide range of habitats in view of their niche specialization. They identified climatic summer water balance, soil texture, and land use as the main environmental parameters differentiating niches in which particular taxa were found. More specifically, the production of long and abundant rhizomes appeared to be an efficient adaptation to poor climatic summer water balance.

5 Major Breeding Achievements

5.1 Meadow Fescue

The majority of the European meadow fescue cultivars have been developed in eastern/central and northern Europe. Meadow fescue germplasm and cultivars have also been used extensively during the last 30–40 years to develop Festulolium hybrids and introgression lines. Meadow fescue is a species with many positive attributes like tolerance toward abiotic and biotic stresses, good persistency and adaptation to grazing and frequent cutting, and high nutritive quality. This makes it highly appreciated in mixtures for conservation cuts and in pastures. It is hard to point at major breeding achievement. It seems that cultivars of meadow fescue are fairly long-lived and that breeding is characterized by a rather slow but continuous improvement; especially for important traits like winter survival and persistency. Casler and van Santen (2000) concluded that considerable progress has been made

in breeding meadow fescue for crown rust resistance. Crown rust resistance has been introgressed from meadow fescue into Italian ryegrass (Armstead et al. 2006).

The common catalogue of varieties of meadow fescue (http://ec.europa.eu/food/plant/propagation/catalogues/agri2009/15.html) in Europe lists 84 cultivars as of May 27, 2009. Casler and van Santen (2000) mentioned that out of 233 *F. pratensis* accessions listed by the Germplasm Resources Information Network (http://www.ars-grin.gov/npgs/, verified August 17, 1999), 79 either had a cultivar name or otherwise appear to be derived from breeding programs. The same authors state that there were four cultivars on the market in USA in 1994, two of them developed in Canada. In USA a meadow fescue cultivar named AM 107 intended for use as turf and overseeding Bermudagrass was granted patent protection in 2008 (see www.nexgenresearch.net/AMF107%20patent.pdf). Meadow fescue is cultivated in 88 regions and republics of former USSR countries, and 38 cultivars have the state permission for utilization (Dzyubenko and Dzyubenko 2009) (www.agroatlas.ru/en/content/cultural/Festuca_pratensis_K/). Also in Japan a number of meadow fescue cultivars have been developed, and they have been bred from material introduced from Europe. Takai et al. (2001) described a new cultivar 'Harusakae', a synthetic composed of eight clones originating from the Scandinavian cultivars 'Boris', 'Leto', 'Tammisto', and 'Salten'. 'Harusakae' has excellent winter-hardiness including freezing tolerance and resistance to *Typhula ishikariensis*. It also contains endophytes of the nontoxic loline alkaloid types. In Sapporo, 'Harusaka' showed 12% higher dry matter yield than the older cultivar 'Tomosakae' in frequent-cutting trials (six cuttings/season).

Induced tetraploidy does not seem to improve agronomic characteristics the same way it does in e.g., *Lolium* species. Autotetraploid meadow fescue has low tillering capacity and Simonsen (1975) found 7% lower yields compared to diploids. However, at least two cultivars have been registered on national cultivar lists, 'Westa' in Poland (abandoned from the list in 2002), and 'Patra' in Latvia (Z. Zwierzykowski, pers. comm.). Spontaneous tetraploids obtained from twin seedlings of diploid cultivars have better tillering capacity than colchicine induced tetraploids, and they have been used extensively as parents in intergeneric crosses within the *Lolium–Festuca* complex (Sulinowski et al. 1982; Zwierzykowski et al. 2006).

5.2 Tall Fescue

Tall fescue was introduced in the USA in the 1800s, but it was not planted to any extent until the release of two cultivars, 'Alta' and 'Kentucky 31' in the early 1940s. 'Alta' is derived from an ecotype population selected on the basis of winter-hardiness, persistence, and ability to remain green during the dry summers of western Oregon. 'Kentucky 31' was released in 1943 and became the most prominent cultivar in the USA. Key characteristics include dependability, adaptability to a wide range of soils, and providing grazing over much of the year. Hundreds of cultivars have been developed subsequently. A list of tall fescue

cultivars released prior to February 2005 is presented in the Tall Fescue Online Monograph (http://forages.oregonstate.edu/is/tfis/book/tables.cfm?section=540 accessed on April 28, 2009). Out of 508 cultivars listed, only 129 are forage type and the rest are turf type. Many of these cultivars were developed with specific characteristics. Adaptation, persistence, and forage yield are the major traits where substantial improvements have been made by breeding. The largest European collection of cultivars with improved characteristics can be found on the French variety list that currently holds 31 cultivars with the oldest one being registered in 1986. This is due to the fact that older obsolete cultivars are outperformed and are withdrawn from the list.

Tall fescue forms symbiotic associations with fungal endophytes. Most tall fescue plants in wild and naturalized stands are endophyte infected. Benefits of endophyte-infected plants include enhanced ecological fitness, improved water and nutrient uptake, and better ability to tolerate grazing in stressful conditions. However, detrimental effects of endophyte-infected plants on livestock performance and metabolism largely restricted tall fescue expansion. Fescue toxicosis in livestock became a major issue which largely modified the breeding objectives. In Argentina and parts of Europe, market entry of endophyte-infected cultivars is restricted by national or recommended lists or other regulations. Most of the early tall fescue cultivars contained wild-type endophytes (E+) and were thus detrimental to animal health. After the 1980s, the breeding target largely moved to developing endophyte-free (E−) cultivars. E− or nontoxic endophyte cultivars can improve animal weight gain by as much as 60–100% when compared to E+ cultivars of tall fescue (Bouton et al. 2002, Hopkins and Alison 2006). In forage agriculture, it is almost impossible to find another technology which can increase animal productivity so dramatically. Persistence is an important issue in E− cultivars. The development of novel endophytes enabled a breakthrough in tall fescue breeding. Plants with the novel endophyte can persist better and are safe for animals. Several cultivars have been developed with the novel endophyte in them (e.g., 'Jesup,' 'Flecha,' 'Baroptima').

5.3 Fine Fescues

Genetic improvement of fine fescues has resulted in the development of more persistent grasses with improved pest and disease resistance, increased stress tolerance, and reduced maintenance requirements (Meyer and Funk 1989). A major improvement has been the incorporation of endophytic fungi into fine fescues (Funk et al. 1994, Saha et al. 1987). Endophytes are considered a liability in forage fine fescues due to the problem of toxicosis in animals associated with alkaloids. On the contrary turf grass breeders prefer to develop new cultivars with a high percentage of endophyte infection due to the benefits associated with them. The presence of these symbiotic fungi provides enhanced stress tolerance and resistance to many insect pests, such as chinch bugs (*Blissus leucopterus hirtus*), bluegrass

webworms (*Parapediasia teterrella*, previously reported as *Crambus* spp.), and billbugs (*Sphenophorus* spp.) (Murphy et al. 1993) as well as diseases including red thread (caused by *Laetisaria fuciformis*) and dollar spot (caused by *Sclerotinia homoeocarpa*) (Bonos et al. 2006).

Improved chewings fescues have better heat and drought tolerance, high seed yield, dark green color, and disease resistance, and are dwarf in growth. The improved cultivars require low maintenance inputs and are tolerant to close mowing (Meyer and Funk 1989). Under high fertility, stand density of Chewings fescue may crowd out other species in mixtures (Smith et al. 1993). Cultivars that show good resistance to summer patch were developed, which otherwise limits the use of some fine fescues under heavy wear or compacted areas.

Significant achievements in strong creeping red fescues include cultivators with larger seeds better seed yield and seedling vigor than Chewings fescues. New cultivars exhibit improved disease resistance, most notably to *Erysiphe graminis* (powdery mildew). Additionally, the improved cultivars have lower turf-type growth habit (Meyer and Funk 1989).

Not much breeding work has been done in slender creeping red fescues, yet these grasses show excellent attributes like salt and heavy metal tolerance that make them ideal for many sites. These grasses show superior winter growth and can tolerate low mowing heights.

Improved hard fescues have their main usage as low maintenance grasses. These are low nitrogen, low water-use turf grasses that require little mowing. They have reduced vertical growth and are resistant to diseases like net blotch (*Drechslera dictyoides*), anthracnose (*Colletotrichum graminicola*), and dollar spot (*Sclerotinia homoeocarpa*) (Meyer and Funk 1989). Hard fescues are slowest among fine fescues to germinate and establish. Incorporation of endophytes into the new material has increased its persistence (Ruemmele et al. 1995).

Sheep fescue is generally used in difficult to mow areas. This species has very good tolerance to drought and low fertility. It can be identified by its fine leaves and blue-green stiff leaves (Beard 1973). Very few cultivars have been developed in this species. Improved cultivars have excellent drought and shade tolerance, fine leaves and stems with abundant basal leaves, early maturity, winter color, and seed head formation.

6 Specific Goals in Current Breeding

6.1 Meadow Fescue

Genetic variation within numerous meadow fescue populations has been documented for forage yield, *in vitro* dry matter digestibility, heading date, resistance to net blotch (*Drechslera dictyoides*), etc. (Aastveit and Aastveit 1989, Frandsen and Fritsen 1982). Aastveit and Aastveit (1989) found that agronomic traits were determined both by additive and non-additive genetic variation. Important traits in

breeding meadow fescue are total dry matter yield and distribution of yield in the season, dry matter digestibility, winter survival and persistency, and disease resistance. A major disease in meadow fescue, which may reduce yield and quality considerably, is net blotch. In addition powdery mildew can also be problematic (Arild Larsen pers. comm.). Leaf diseases usually cause injury in later cuts and especially in seed fields. Net blotch is a very serious disease in seed production fields and the prevalence of this disease has increased in recent years in Norway (Havstad 2009). Meadow fescue is generally quite resistant against the low-temperature fungi snow mould (*Microdocium nivale*), gray snow mould (*Typhyla incarnata*), speckled snow mould (*Typhula ishikariensis)*, and sclerotinia snow mould (*Sclerotinia borealis*). Bacterial wilt (*Xanthomonas campestris* pv. *graminis*) is a serious disease and considered the main reason for shortened persistence of meadow fescue and ryegrasses in Switzerland (Michel 2001). Enhanced resistance to *Xanthomonas* is sought by using artificial inoculation, and an improvement by recurrent selection was reported, although progress leveled off after four to six cycles of selection (Boller et al. 2001).

Endophytes have not been paid as much attention in meadow fescue as in tall fescue and the ryegrasses. Huizing et al. (1991) showed that eczema in lambs grazing meadow fescue in the Netherlands was linked to endophyte infestation. Holder et al. (1994) found that 23 out of 150 meadow fescue accessions stored at GRIN were infected with the endophyte *Neotyphodium uncinatum.* Recently, *Neotyphodium* endophyte infection among-and-within seven Nordic cultivars of meadow fescue (two Norwegian and five Finnish) was studied (Saari et al. 2009). Very variable degrees of infection were observed from complete endophyte free (Norwegian 'Fure') to nearly completely infected (Finnish 'Inkeri'). They also demonstrated that endophyte infection (Loline) increased resistance against the bird cherry oat aphid (*Rhopalosiphum padi* L.) which can transmit barley yellow dwarf virus. A survey of endophyte infection in eight populations of meadow fescue from Italy showed 100% infection with endophytes but no ergovaline production (Jensen et al. 2007). Leuchtmann et al. (2000) determined levels of alcaloids in different grass-endophyte associations and found no ruminant toxic ergovaline or peramine in *Neotyphodium uncinatum* infected meadow fescue breeding populations and cultivars such as 'Preval' and 'Pradel' (FP1 and FP5), while levels of protective lolines were similar to, or higher than those in *Neotyphodium coenophialum* infected tall fescue. More emphasis on endophytes in meadow fescue breeding is needed.

6.2 Tall Fescue

Tall fescue became popular in areas where it was introduced. Thus, apart from high forage yield, adaptation to a specific environment has also been a major breeding goal for tall fescue breeders. Continental germplasms are generally winter-hardy, but cannot withstand hot and dry summers. Drought tolerance or avoidance (summer dormancy) is an important breeding goal for Continental tall fescue. Drought

tolerance is a complex trait that involves many tolerance and avoidance mechanisms. Attempts were undertaken to improve drought tolerance in tall fescue through: (i) selection for decreased carbon isotope discrimination; (ii) improved root characteristics and mass; (iii) improved aluminum tolerance; and (iv) osmotic adjustments. Mediterranean tall fescue is well adapted to harsh summers, but is usually susceptible to cold weather. Winter-hardiness would be expected to expand its cultivation area. Tall fescue in pastures in the USA is mainly used for grazing animals. Grazing tolerance is an important objective in tall fescue breeding. Selection of plants exposed to heavy or continuous grazing is an effective way to identify grazing-tolerant genotypes. Space planted and seeded sward plots are equally effective in determining persistence of populations under heavy grazing pressure (Hopkins 2005). Forage yield under cutting is also an important selection criterion for European, Australian, and Argentinean conditions.

Forage quality is an important breeding goal as a slight increase in digestibility has been shown to have a major positive impact on animal performance (Casler and Vogel 1999). Traits such as forage and seed yield, *in vitro* dry matter digestibility (IVDMD), and detergent fiber parameters show promise with distinguished additive genetic variations (Nguyen et al. 1982, Nguyen and Sleper 1985). Protocols have been developed to determine the *in vitro* dry matter digestibility (IVDMD). In addition selection is performed on palatability since the correlation between digestibility, palatability, and animal performance is decreasing in elite material (D. Noel pers. comm.). Good cultivars for Europe should have a combination of good forage yield distribution throughout the year, persistency, high quality (defined by soft leaves, good digestibility, and high palatability), in combination with good disease resistance, fast establishment, and good seed yield. As regards forage quality, methods for measuring *in vitro* dry matter digestibility are laborious and time consuming. Molecular marker-assisted breeding has been initiated at the Noble Foundation to develop tall fescue cultivars with high digestibility (Saha et al. 2007). Transgenic tall fescue plants with lower lignin content and higher forage digestibility have been developed (Chen et al. 2004). Seasonal distribution of forage yield is as important as the total yield. Increased fall and winter forage yield is considered highly desirable in the USA. Increased concentration of Ca, P, and Mg was reported in selected populations (Sleper et al. 2002). Low mowing height and shading tolerance are always the priority traits in turf-type tall fescue breeding.

Stem rust caused by *Puccinia graminis* Pers.:Pers. subsp. *graminicola* Z. Urban is considered the major disease of tall fescue, especially for a seed crop. Germplasm with a significant level of rust resistance have been identified (Barker and Welty 1997). However, current commercial cultivars grown for seed can only be produced reliably with chemical protection. Resistance to defined isolates of stem rust is considered a qualitative trait which is mainly controlled by a single or few genes. Two cycles of recurrent selection for resistance to stem rust demonstrated improved seed yields when substantial disease pressure occurred (Barker et al. 2003). Gray leaf spot disease [caused by *Magnaporthe grisea* (T.T.Hebert)] is an increasingly severe disease of turf-type tall fescue in the USA. It is now a persistent problem in the southeast USA. This pathogen is also the causal agent of rice blast. Partial

resistance to gray leaf spot was identified in *L. perenne*, and quantitative trait loci associated with disease resistance were mapped (Curley et al. 2005). Brown patch disease (caused by *Rhizoctonia solani*) is the most severe disease in tall fescue lawns under warm and humid weather conditions. Attempts have been made to identify QTL associated with brown patch resistance in tall fescue (Jonathan Bokmeyer pers. comm.). Transgenic tall fescue plants resistant to both gray leaf spot and brown patch disease were identified (Dong et al. 2008). Other important diseases in Europe are *Xanthomonas* and *Drechslera*.

6.3 Fine Fescues

Breeding goals in fine fescues depend on whether the focus is on forage or on turf use. For example, turf breeders may want high fibre content because it improves wear tolerance whereas forage breeders want low fibre content as it is associated with improved digestibility (Vogel et al. 1989). Most breeding efforts in fine fescues are targeted to turf use. General breeding objectives for turf use such as resistance to wear and tear, sod forming ability, or lawn quality aspects are discussed in Chapter 6 of this volume. Here, we focus on resistance to diseases.

Endophytes confer resistance to many pests and diseases. Studies have shown that fine fescues have consistently exhibited endophyte-mediated suppression of dollar spot (*Sclerotinia homoeocarpa*) and red thread (*Laetisaria fuciformis*) diseases when compared with closely related endophyte-free entries (Bonos et al. 2006, Clarke et al. 2006). Therefore turfgrass breeders usually prefer to develop new cultivars with a high percentage of endophyte infection (Meyer and Funk 1989).

Susceptibility to diseases is considered to be one of the weaknesses of fine-leaved fescues (Ruemmele et al. 2003). Some of the diseases that fine fescues are susceptible to are leaf spot (*Drechslera* spp.), Fusarium patch [*Microdochium nivale* (telemorph *Monographella nivalis*) (Schaffnit) E. Muller], take-all patch [*Gaeumannomyces graminis* (Sacc.) Arx & D. Olivier vat. *Avenae* (E.M. Turner) Dennis], powdery mildew (*Erysiphe graminis* DC), brown patch (*Rhizoctonia solani* Kiihn), red thread, gray snow mold *(Typhula* spp.), pythium blight (*Pythium* spp.), dollar spot, and stripe smut [*Ustilago striiformis* (Westend.) Niessl] (Meyer and Funk 1989). Summer patch (*Magnaporthe poae* Landschoot and Jackson) can be a serious problem in moderate-maintenance areas (Kemp et al. 1990). Although grass breeders have made significant improvements in developing many disease resistance cultivars, stable genetic resistance is needed in new cultivars. Hence breeding for disease resistance should be an integral part of grass breeding programs (Vogel et al. 1989). Although genetic sources of resistance have been reported for many diseases of economic importance (Braverman 1986), the inheritance of disease resistance needs to be studied more thoroughly. Disease resistance in the major agronomic cereal crops has received significant attention and relatively little research to date has been reported on in many grass species (Bonos et al. 2006). Therefore, results from arable crops of *Poaceae* may be useful in breeding for disease resistance in fine fescues.

7 Breeding Methods and Specific Techniques

All fescues are highly cross-pollinated and self-compatibility varies among species (Schmit et al. 1974), meadow fescue and tall fescue being highly self-incompatible (Lundqvist 1962, Xu et al. 1991). Consequently, these species exhibit high heterogeneity among individuals within and among populations. Hence breeding procedures developed for cross-pollinated species, i.e., methods that increase the frequency of favorable alleles in a population, are mostly applicable for fescue cultivar development. Cultivars of fescue species are either improved populations developed by phenotypic mass-selection of ecotypes or a breeding population, or synthetics. Synthetics are usually constructed from parental clones or remnant seed following progeny testing using half-sib (HSF) or full-sib families (FSF), and this is sometimes combined with among-and-within family selection (AWF) (Casler and Brummer 2008). Phenotypic evaluation of clones has been and is being used in fescue breeding. Clonal selection is usually effective for traits with high heritability, like heading date and disease susceptibility, but is unlikely to be effective for traits demonstrating genotype × environment interactions like herbage yield and persistency. Single clone selection has improved the selection for disease resistance in several turfgrass species including fine fescues. These plants are typically maintained for 1–2 years at a height of 5 cm before superior clones are selected for further improvement based on turf quality (i.e., fine leaf texture, high shoot density, dark green color, clean mowing quality) and the absence of disease (Bonos et al. 2006).

Ecotype selection was the earliest method used to develop fescue cultivars and is still considered an important breeding method (Fjellheim and Rognli 2005a). Germplasms from old pastures, roadsides, coastal areas, mining sites, seed fields, etc., were collected and tested in a number of environments; seed increased, and finally cultivar(s) released. Most of the earliest fescue cultivars were developed in this manner (Hopkins et al. 2007). The oldest Nordic, and possibly also European, cultivar, 'Svalöfs Sena' from Sweden, dates back to 1917 and some of the newer cultivars are still developed from ecotypes (Fjellheim and Rognli 2005a). Cultivars from ecotypes are either developed directly from multiplication of ecotypes or more commonly by phenotypic mass selection. Ecotype selection capitalizes on natural selection and together with the high levels of genetic variation present in the germplasm, can act to generate new populations in short periods of time (Valay and Van Santen 1999). As an example, the most recent Norwegian cultivar 'Norild' (Figure 4) is a synthetic cultivar based on 11 clones selected among surviving plants of half-sib (HSF) families created from a local population tested for 3 years at a very northern location (70°N). Molecular diversity studies have shown that this cultivar is very different from the local population it originates from and also that it is the least diverse of all Nordic cultivars studied (Fjellheim and Rognli 2005a). This case demonstrates strong selection being imposed, most probably for winter survival, which has reduced the within population variation considerably.

Recurrent phenotypic selection is a popular breeding technique to develop improved cultivars. Superior genotypes with desirable trait(s) are selected from a

Fig. 4 Performance trial carried out at Vågønes, Bodø, Norway (67.3°N). Meadow fescue cultivar 'Norild', developed from a local Nordic population, and the Dutch cultivar 'Stella' show excellent winter survival when compared to a series of *Festulolium* checks (Photo B. Volden)

population or diverse germplasm, the selected plants are intermated in isolation, and this cycle is repeated for multiple generations. Recurrent selection appears to be a powerful means of accumulating favorable alleles in a population and is often the method of choice for improving traits with low heritability. It can give considerably uniform cultivars from diverse germplasm.

8 Integration of New Biotechnologies in Breeding Programs

Although detailed linkage maps are not available for many of the fescue species, saturated molecular linkage maps are available for almost all the major cereal crops. This information can be used in identifying candidate genes for the trait of interest that can be further used in a marker-assisted selection program. A loose association of marker and trait could be used in discarding the undesirable plants allowing the breeders to test only the superior genotypes (Brummer 1998). Association and comparative mapping has been extensively studied in major cereal crops and major forage crops. As mapping studies in many fescue species like the fine fescues are not widely reported, inferences from studies in tall fescue and meadow fescue and the cereals can be utilized in fine fescue breeding programs.

Considering its genetic complexity and the associated difficulties encountered by conventional breeding, biotechnology is claimed to offer many alternative and effective strategies to improve forage and turf grass cultivars (Spangenberg et al. 1998). Transgenic approaches may supplement conventional breeding, since they offer the opportunity to generate unique genetic variation that is either absent or has very low heritability (Wang and Ge 2006). Genetic transformation either by direct gene transfer to protoplasts, microprojectile bombardment or by, *Agrobacterium* has been reported in forage and turf grasses including red fescues. With the development of novel gene transfer techniques for forage and turf grass species, generating transgenic plants with improved agronomic characters or evaluating novel strategies for grass improvement is under way on a large scale (Wang and Ge 2006).

8.1 Genomic Resources in *Festuca*

Substantial interspecific variation in nuclear DNA content in *Festuca* which cannot be accounted for by variation in chromosome numbers was described by Seal (1983). It was also found that polyploid fescue species may have lost DNA since their divergence from progenitor species of lower ploidy levels. The DNA content of hexaploid tall fescue was estimated to about 5.83 pg per haploid genome corresponding to a genome size of 5.7×10^9 bp (Seal 1983). However, the nuclear DNA content of tall fescue lines/cultivars in a recent diversity study varied from 7.6 to 10.6 pg with a mean of 9.6 pg per haploid genome (M. Saha unpublished data). The DNA content of meadow fescue is about 1.9 pg per haploid genome corresponding to a genome size of 1.86×10^9 bp (Seal 1983). About 46,000 tall fescue Expressed Sequence Tags (ESTs) are available in public databases (http://www.ncbi.nlm.nih.gov/ accessed on April 12, 2009). A total of 5,320 genomic sequences derived from $(GA/CT)_n$ enriched genomic libraries of KY-31 pool plants are also maintained at the NCBI. ESTs have become a cost effective, time efficient, and unique source of SSR markers (Eujayl et al. 2004, Saha et al. 2004). About 1.3% of tall fescue ESTs contain SSRs which are suitable for marker development. Tall fescue ESTs developed at the Noble Foundation (43,000) have been used to develop 780 microsatellite primer pairs. A total of 511 primers have been developed from the genomic sequences (Saha et al. 2006). In addition, stress-related gene sequences from other species were used to identify the orthologous tall fescue sequences from which sequence tagged site (STS) markers were developed. A bacterial artificial chromosome (BAC) library of *F. pratensis* was developed at IBERS, Aberystwyth (Donnison et al. 2005). It has been used in comparative genomic strategies to clone candidate orthologous sequences to the CONSTANS-like rice Hd1(Se1) gene in *Lolium perenne* and *Festuca pratensis* (Armstead et al. 2005), and to isolate orthologous sequences for frost tolerance and lignin biosynthesis candidate genes in *F. pratensis* (Rudi et al. manuscript). Also high-throughput EST sequencing of meadow fescue to study differential gene expression under cold acclimation and to generate SNP markers is underway in Norway.

A Diversity Arrays Technology (DArT) array for five grass species, *F. pratensis*, *F. arundinacea*, *F. glaucescens*, *L. perenne,* and *L. multiflorum*, has been developed as a global collaboration funded by The Czech Republic (Kopecky et al. 2009a). The DArTFest array contains 7,680 probes derived from methyl-filtered genomic representations of the five species. In a first marker discovery experiment using about 40 genotypes of each species, 3,884 polymorphic markers were detected, varying from 821 to 1,852 for each single genotype. To test the usefulness of DArTFest array for physical mapping, DArT markers have been physically mapped to each of the seven chromosomes of *F. pratensis* using monosomic and disomic chromosome substitution lines of *F. pratensis* into *L. multiflorum* (Kopecky et al. 2008), and to chromosome bins on these chromosomes. The DArTFest array will facilitate the development of genetic maps in *Festuca* and *Lolium*, analyses of genetic diversity, and monitoring of the genomic constitution of *Festuca* × *Lolium* hybrids. It could

also be instrumental in marker-assisted selection for multiple traits or for specific genome regions.

At IBERS, UK, a set of monosomic substitution lines, each carrying one *F. pratensis* chromosome and 13 *L. perenne* chromosomes, have been developed (King et al. 1998), and backcrossed to *L. perenne* to create series of recombinants with introgressed segments of *F. pratensis* chromosomes (King et al. 2007a). This is a very useful genomic resource that is being utilized to develop physical maps by 'introgression mapping' (King et al. 2007a). European groups interested in genomic research of forage and amenity grasses have established an initiative called ELFIN – European Lolium and Festuca Initiative (www.elfin-initiative.co.uk/). The goal is firstly to develop a physical map of the grass genome (*L. perenne*), an effort that now is underway in UK, exploit this resource for determination of the genome location of genes controlling target traits, and finally to obtain the complete genome sequence.

8.2 Molecular Markers and Their Application

In the past decade, a comprehensive molecular marker system with more than 1,800 primers has been developed for tall fescue and used for genetic diversity analysis, construction of genetic linkage maps, QTL analyses, and marker-assisted breeding at the Noble Foundation, USA. In the early 1990s, attempts were undertaken to generate restriction fragment length polymorphism (RFLP) markers for tall fescue. Subsequently, randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers were developed and used for genetic analysis of tall fescue. Many of these markers were also used to construct genetic linkage maps of tall fescue (Saha et al. 2005), ryegrass [Italian (*L. multiflorum* Lam.) × perennial (*L. perenne* L.)], and bentgrass (*Agrostis stolonifera* L.). In meadow fescue, RFLP markers were developed from a genomic DNA library and used in combination with AFLP and a few isozyme markers to develop the only linkage map available for meadow fescue (Alm et al. 2003).

Development of intron-flanking EST markers for the *Lolium*/*Festuca* complex using rice genomic information was reported by Tamura et al. (2009). Primer sets were designed from Lolium/Festuca ESTs that showed high similarity to unique rice genes and used to amplify insertion-deletion (indel)-type markers and cleaved amplified polymorphic sequence (CAPS) markers that could distinguish between *Lolium perenne* and *Festuca pratensis*. Many indel-type markers had high species specificity, and 15 markers were completely specific to both species. Forty-nine of the CAPS markers completely distinguish between the two species at bulk level. Such markers are very valuable tools in breeding of *Festulolium.*

8.3 Genetic Linkage Maps and Marker-Assisted Selection

The construction of a genetic linkage map of meadow fescue using a full-sib family of a cross between a genotype from a Norwegian population (HF2) and a genotype

from a Yugoslavian cultivar (B14) was reported by Alm et al. (2003). They used the two-way pseudo-testcross procedure to develop separate maps for each parent, as well as a combined map. The combined map consisted of 466 markers, RFLPs and AFLPs, and a few isozymes and SSRs, with a total length of 658.8 cM with an average marker density of 1.4 cM/marker. Due to the mapping of many heterologous cereal RFLP anchor probes, it was possible to conduct relatively detailed comparisons of the structure of the chromosomes of meadow fescue and those of the Triticeae species and *Lolium*. A high degree of orthology and colinearity was observed between meadow fescue and the Triticeae genome(s) for all linkage groups, and the authors proposed to designate the individual linkage groups 1F–7F in accordance with the orthologous Triticeae chromosomes. As expected, the meadow fescue linkage groups were highly orthologous and colinear with Lolium. Studies of chromosomal rearrangements relative to Triticeae and rice showed that the meadow fescue genome has a more ancestral configuration than any of the Triticeae genomes. This is especially evident in chromosome 4F which is completely orthologous to rice chromosome 3 in contrast to the Triticeae where this rice chromosome is distributed over homoeologous groups 4 and 5 chromosomes. Recently, the meadow fescue map was enriched with more than 200 DArT markers (Kopecky et al. 2009b). A peculiar feature of the meadow fescue map is that the combined maps for chromosomes 2F and 3F derive completely from segregation in the female parent. It was not possible to detect segregation of any markers on chromosomes 2F and 3F of the male parent HF2/7.This was also the case with the new DArT markers (Simen R. Sandve unpublished). The most likely reason for this anomaly is that both chromosomes carry more or less complete chromosomal inversions and that the male parent is heterozygous for these inversions. The male parent originates from the old local cultivar 'Løken' and it is interesting that cytogenetic studies in this cultivar detected a very high frequency (42%) of inversion heterozygotes (Simonsen 1975). The presence of inversion heterozygotes are classically explained as co-adapted gene complexes being favored by natural selection since recombination within the inversions gives non-viable gametes. The presence of co-adapted gene complexes is supported by the introgression mapping of *Festuca*/*Lolium* at IBERS (King et al. 2007b). They demonstrated that a substantial component of the coding sequences in monocots is localized in regions of very low and even negligible recombination. The findings indicate that large supergene (co-adapted) complexes that confer a selective advantage to the individual have been favored during evolution of the grass genomes.

The first genetic linkage map of tall fescue was constructed using a F_1 population developed by crossing HD28-56, a plant with high forage quality, to a selected plant of 'Kentucky-31' following the two-way pseudo-testcross procedure (Xu et al. 1995). The map covered 1,274 cM on 19 LGs with an average of five loci per LG and a marker density of 17.9 cM per marker. An attempt was made to develop a detailed PCR-based genetic linkage map of tall fescue (Saha et al. 2005). A mapping population was constructed by crossing HD28-56 to R43-64, a genotype from the Oklahoma local collection. A total of 773 AFLP and 343 microsatellite markers were used to construct the linkage maps following the pseudo-testcross strategy. Each parental map was first constructed followed by bi-parental consensus

maps. The female (HD28-56) map included 558 loci placed in 22 LGs and covered 2,013 cM of the genome. The male (R43-64) map comprised 579 loci grouped in 22 LGs with a total map length of 1,722 cM. The marker density in the two maps was 3.61 cM (female parent) and 2.97 (male parent) cM per marker. The consensus map covered 1,841 cM on 17 LGs, with an average of 54 loci per LG, and had an average marker density of 2.0 cM per marker. Six of the seven predicted homoeologous groups were identified. High levels of segregation distortion are very common in outcrossing polyploids. About 23% of markers showed segregation distortion in this mapping population. Markers with significant segregation distortion clustered in four of the LGs of the consensus map. This indicated that some specific genomic regions are mainly responsible for segregation distortion. Considering the practical utility of markers in a marker-assisted breeding program, a microsatellite map of tall fescue was also developed (Saha et al. 2007). The same mapping population described earlier was used for map construction. This map was used to identify molecular markers associated with traits of interest and to map quantitative trait loci (QTL).

Marker-assisted selection (MAS) to improve economically important traits of tall fescue can expedite the breeding cycles (Xu et al. 1995). With appropriate markers, a large number of plants can be screened quickly at an early stage, thereby reducing field experiment size, labor requirements, and time. Increased digestibility and superior stem rust resistance are target traits in several breeding programs. Phenotypic data on IVDMD have been collected from the mapping population for three consecutive years. The population was also evaluated for stem rust resistance under both greenhouse and field conditions. Data obtained from these experiments were evaluated in conjunction with SSR marker data in order to identify possible QTLs associated with traits of interest. Microsatellite markers associated with forage digestibility and stem rust resistance in tall fescue were identified. Seven markers associated with high digestibility and six others linked to low digestibility were used to initiate MAS (Saha et al. 2007). In addition, six markers associated with stem rust resistance and susceptibility were used in a MAS program (unpublished data). It would be useful to select for alleles leading to increased digestibility/resistance while at the same time eliminating alleles contributing to decreased digestibility/susceptibility. Verification of selected plants for the desired traits is in progress in field evaluations. A population developed through MAS will be evaluated in comparison with populations based on phenotypic selection. Preliminary results suggest promise of MAS in tall fescue.

QTLs for frost and drought tolerance, and for winter survival in the field, have been mapped in meadow fescue using the 'B14/16 × HF2/7' mapping family (Alm 2001). A total of 13 chromosomal regions were found to be involved in determining stress tolerance in this population. Major QTLs for frost tolerance/winter survival were located on chromosomes 1, 2, 5, and 6, and for drought tolerance traits on chromosomes 1, 3, 4, and 5. QTLs for several of the stress tolerance traits mapped to the same regions on *Festuca* chromosomes 1F, 4F, and 5F. The locations of two frost tolerance/winter survival QTLs on chromosome 5 correspond to those of the Fr-A1

and Fr-A2 on wheat homoeologous group 5A. The coincident location of several stress tolerance QTLs in *Festuca* with QTLs and genes mapped in Triticeae species, notably two frost tolerance QTLs on chromosome 5 and dehydrin and CBF transcription factor genes, indicate conserved genes involved in stress tolerance across the grasses. Fang (2003) found a total number of 34 chromosomal regions containing QTLs for seed yield and component traits in meadow fescue. QTLs for a number of related traits clustered in a few chromosomal segments, most evident on linkage groups 1F, 4F, and 5F. This indicates that there must be one or a few major gene(s) in these regions that affect reproductive development with pleiotropic effects on many traits. Concurrent QTLs for panicle fertility and seed yield were detected on chromosomes 1F, 2F, 4F, and 6F, and these should be interesting for the future development of molecular markers for improved seed yield. Comparisons of the QTL positions with positions of QTLs of identical or similar traits in other grass (cereal) species, using common anchor markers, identified a number of putatively orthologous QTLs. Mapping of an orthologue of the wheat vernalization gene *Vrn1* in meadow fescue (Ergon et al. 2006) and its association with vernalization and seed yield related traits demonstrate conservation across grass species and the value of comparative genomics approaches. In addition the meadow fescue mapping population has been utilized to map herbage quality traits (Solberg 2002) and segregation for stem rust resistance was recorded in a field study of the mapping family at Lusignan, France.

8.4 Transgenics

Transgenesis provides the most direct means of introducing truly novel traits to crop plants. Transgenic tall fescue development was initiated by direct gene transfer to protoplasts (Wang et al. 1992). Later, biolistic transformation was used to develop transgenic tall fescue (Cho et al. 2000). Embryogenic cultures were used to produce transgenic tall fescue plants and this has become a useful method (Wang et al. 2001). A major problem of biolistic transformation as opposed to *Agrobacterium*-mediated transformation is that the former often produces multi-copy transformants (Wang et al. 2003a). After successful application in other monocots, an *Agrobacterium*-mediated transformation protocol has been widely adopted for the production of transgenic tall fescue (Dong and Qu 2005).

Improving forage quality and abiotic stress tolerance are the key targets of transgenic tall fescue breeding, and transgenic tall fescue plants have been produced with the aim of improving forage digestibility (Chen et al. 2003, Chen et al. 2004). Lignin is a key component of the cell wall, and lignification of plant cell walls is largely responsible for lowering forage digestibility. Cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT), two key genes involved in the lignin biosynthesis pathway, were cloned and characterized in tall fescue. Transgenic plants with reduced lignin concentration, altered lignin composition, and increased dry matter digestibility (7.2–10.5%) have been developed (Chen et al. 2003, Chen et al. 2004).

Protein quality and content are other determinant factors for forage quality. Methionine and cysteine are among the most essential amino acid components of protein that largely influence wool growth in sheep. Transgenic tall fescue plants with the sunflower seed albumin gene were stably integrated (Wang et al. 2001). However, the corresponding sulphur-rich SFA8 protein did not accumulate to the level necessary to make a significant impact on ruminant diets. Overexpression of *AtNHX1* gene improves salt tolerance in transgenic tall fescue (Tian et al. 2006). The transgenic plants showed remarkable salt tolerance compared to control plants. The bacteriophage T4 lysozyme gene confers resistance to both gray leaf spot and brown patch diseases in transgenic tall fescue plants (Dong et al. 2008). Transgenic turf-type tall fescues were developed which showed better tillering ability, higher chlorophyll a and b levels, and greater cold tolerance than the non-transgenic checks. The transgenic plants were vigorous and remained green even under low temperature conditions (Hu et al. 2005).

Transgenic plants are usually developed under controlled environments. Field performance of transgenic and tissue culture regenerated plants was generally inferior to that of the seed-derived plants. However, no major differences were found on performances between the progenies of transgenic plants and the progenies of seed-derived plants (Wang et al. 2003b). Although primary transgenic plants had various levels of pollen viability, progenies of transgenic plants (both T_1 and T_2 generation) showed similar pollen viability when compared with that of seed-derived plants (Wang et al. 2004). Pollen is an important vector of transgene flow in outcrossing grasses and thus is considered an important aspect of risk assessment in a transgenic production system. Isolation by various distances depending on generation is well established in seed multiplication schemes of outbreeding grasses in order to maintain cultivar purity. In general, the highest rates of gene flow can be expected in wind-pollinated, semi-domesticated crops such as forage and pasture grasses, which are cross-compatible with wild or weedy populations of the same species (Rognli et al. 2000). This makes it very challenging to commercialize transgenic forage grasses. The costs of meeting regulatory requirements and market restrictions are substantial impediments to the commercialization of transgenic crops. In order to provide baseline data for risk assessment of gene flow in a typical outcrossing grass species, Rognli et al. (2000) conducted a donor–acceptor pollen dispersal experiment in meadow fescue using an isozyme marker. Gene flow was shown to decrease rapidly with distance to the donor field up to 75 m, and beyond this distance much more slowly. The ability of donor pollen to fertilize acceptor plants depended very much on the density of the acceptor plants.

9 Seed Production

Seed production is one of the important steps in plant breeding since the commercial value of a cultivar is often determined by its seed yield capacity (Rognli 2007). All fescue species have a dual flower induction requirement; they need

vernalization and/or short days in the autumn to initiate reproductive development, and long days and moderate temperatures for stem elongation and flowering in the spring (Heide 1994). Seed production is a special-purpose crop which requires proper species-specific management practices, e.g., autumn fertilization and straw burning, and seed yields can be quite variable depending on variation in soil, rainfall, temperature, photoperiod, plant pests, etc., between locations. Average seed yield of tall fescue in the US varies from 550 to 1,800 kg ha^{-1}. Seed yield jumped from 1,210 to 1,765 kg ha^{-1} in commercial production fields in Oregon, USA, from 1994 to 2004 (Hopkins et al. 2007). This increase in seed production can be attributed to genetic gain, given the strong emphasis on selecting turf types with increased seed yield. Tall fescue produces abundant seed in the transition zone of the USA; this is considered an additional income source to the growers. However, in the USA, the Oregon Valley is famous for grass seed production. A total of 118,948 tons of forage and turf-type tall fescue seed was produced from 70,650 ha in 2008 (http://cropandsoil.oregonstate.edu/seed-ext/FnF.html, accessed on April 28, 2009). The seed production increases for tall fescue in Europe are less spectacular mainly because cultivars cannot be released commercially before they have been listed on an official variety list that is comparing new cultivars with available ones based on agronomic characteristics. Economic seed yield is not (yet) playing any important role in the decision making process to release new cultivars.

There are few published records of seed yields of meadow fescue in Europe. In general meadow fescue is considered a good seed producer and a quite easy crop to manage. In Saxony, one of the main European production areas, in the period 1998–2007, average saleable seed yields of meadow fescue on a total surface of 1500–2500 ha varied between 660 and 950 kg ha^{-1} and were very similar to those of perennial ryegrass (Schaerff 2008). The average seed yields in Norway of cultivars 'Salten' and 'Fure' were 541 and 672 kg ha^{-1}, respectively, in the period 1989–1996, whereas average seed yield in Denmark was 906 kg ha^{-1} (Havstad 1998). This difference is probably due to a higher seed yield potential of the main Danish cultivar and not due to a more favorable climate for seed production (Havstad 1998). Generally, little selection has been exerted on seed yield and seed yield components in forage grasses. Therefore, genetic variation and heritability for these traits are large (Rognli 2007). Fang et al. (2004) studied phenotypic and genotypic variation for seed yield and related traits in a full-sib family of meadow fescue (*Festuca pratensis* Huds.) grown at two locations in Norway. Their estimates of broad sense heritabilities (h^2) for the traits were highest (0.80) for seed yield per plant, and seed yield per plant and reproductive components like seed weight per panicle and fertility exhibited the largest genotypic coefficients of variation (GCV %), being around 34%. Fang et al. (2004) also conducted a path coefficient analysis of seed production components in meadow fescue. The path analysis showed that fertility was the most important component trait contributing to seed yield. Since panicle fertility is highly correlated with seed weight per panicle, this component trait could be used in selection for seed yield. The path analysis also demonstrated that flag-leaf width had an important direct effect on seed yield and indirectly through panicle fertility. This indicates that large flag leaves contribute to a good seed-set (panicle

fertility) through assimilate reallocation via the stems to the inflorescence in the period of anthesis, and that this contributes to higher seed yields. The importance of the flag-leaf for grain yield in cereals is well-known, and it is not surprising that this is the case also in grasses. The importance of compensation among seed yield components has been demonstrated in a study of the two cultivars 'Fure' and 'Kalevi' which have contrasting growth habits (Makela and Kousa 2009). Although 'Kalevi' had significantly more panicles than 'Fure', the two cultivars gave about the same seed yield because 'Fure' compensated for the lower number of panicles with increased panicle size. Good compensating ability among seed yield components is valuable in relation to a combined breeding for high herbage yield and quality and good seed yield capacity.

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Festulolium

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1 Introduction

Festulolium refers to natural or synthetic intergeneric hybrids between obligate outbreeding species of the *Festuca* (fescue) and *Lolium* (ryegrass) genera, species considered frequently as ideal components of agricultural or turf-grass systems. Intermediate forms between the two genera have long been recognized in nature and considered as hybrids (× *Festulolium* spp.) by taxonomists (e.g., Hubbard 1992) mostly on the basis of the inflorescence shape and their suspected progenitor species' combinations.

The first synthetic *Festulolium* hybridization was reported by Jenkin (1933) and involved perennial ryegrass (*L. perenne* L.) and meadow fescue (*F. pratensis* Huds.) which also exists as a natural hybrid (× *F. loliaceum* Hudson (P. Fournier). However, the commercial breeding of *Festulolium* cultivars developed much later and initially through two alternative pathways: by introgression of *Lolium* spp. into 6x tall fescue (*F. arundinacea* Schreb.) in the USA (Buckner et al. 1977, 1983) and through amphiploidy in Europe (Lewis et al. 1973) where by intercrossing autotetraploids, the intact genome of *Lolium* spp. was combined with that of *F. pratensis* to encourage preferential homologous chromosome pairing and to maintain and stabilize chromosome composition across generations.

In the European Union (EU), all hybrid cultivars derived from 6x *F. arundinacea* (formally *F. arundinacea* var. *genuina*, $2n = 6x = 42$) were classified into tall fescue national lists while the *Festulolium* definition was formally restricted only to the *L. multiflorum* × *F. pratensis* (= × *F. braunii* (K. Richter) A. Camus) hybrid combination (66/401/EEC & 92/19/EEC directives). Recently (2004/55/EC), the European Commission extended the definition of *Festulolium* 'to include all hybrids resulting from the crossing of a species of a genus *Festuca* with a species of a genus

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Lolium' regardless of chromosome number and whether hybrids were intentionally backcrossed in parental species. Consequently, *Festulolium* may at present include any amphiploid or introgression line derived from any *Lolium* × *Festuca* hybrid combination.

2 Objectives and Strategies in Current *Festulolium* Breeding

Lolium and *Festuca* share many complementary characters (Thomas and Humphreys 1991). In general, *L. perenne* and *L. multiflorum* (Italian ryegrass) are considered the optimal species for grassland agriculture as they provide high yields of nutritious forage. However, they lack resilience against abiotic stresses and it is primarily for this reason, with summer and some winter stresses likely to increase due to climate change, those genes are being sought from more robust and stress-adapted *Festuca* species (Yamada et al. 2005). *Lolium* and *Festuca* species, including those of prime importance in forage agriculture (e.g., *L. perenne* and *F. arundinacea*) are also employed in the turf-grass industry and as amenity grasses. Target traits for turf-grass breeding include improved nitrogen and water-use efficiency and the advances being made currently in developing these traits in forage agriculture can be applied similarly to the turf grasses. Combining *Lolium* and *Festuca* traits by interspecific hybridization relies upon two distinct and contrasting genetic strategies, amphiploidy and introgression, in both cases replicating in synthetic hybrids and their offspring, strategies found widely among natural interspecific and intergeneric hybrids.

2.1 Amphiploidy

Improvement by way of amphiploidy is achieved by accumulating the phenotypic effects of parental species and is obtained by maintaining in hybrids intact parental genome sets and securing their balanced transfer over any subsequent generations. For this reason, *Festulolium* cultivar development thus far, has largely been through the amphiploidy approach.

Festuca species (except *F. pratensis*) are polyploid comprising homoeologous genome sets. Despite their incomplete homology, homoeologous *Festuca* chromosomes do pair preferentially, and this is encouraged in amphiploid *Festulolium* hybrids by the greater chromosome pairing affinity of the disomic sets of homologous *Lolium* chromosomes that restrict instances of intergeneric chromosome pairing (Zwierzykowski et al. 2008) (Figure 1).

Although genome inheritance in *F. arundinacea* (and in *F. glaucescens* – formally *F. arundinacea* var. *glaucescens*, $2n = 4x = 28$) was shown to be of disomic nature with homologous pairing under genetic control (Jauhar 1975), it is hemizygous ineffective. Strict disomic inheritance is not fully achieved in any existing *Festuca* × *Lolium* amphiploid cultivars where genome balance may consequently

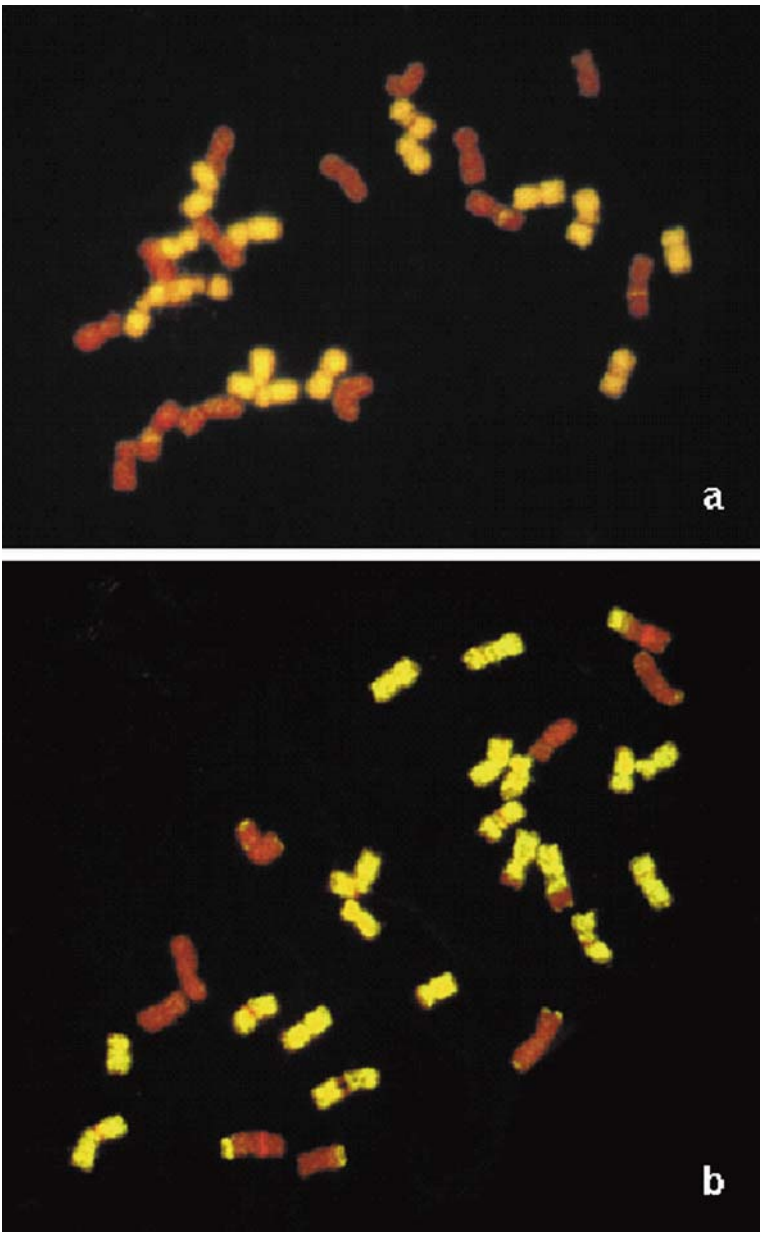

Fig. 1 Genomic in situ hybridization (GISH) on mitotic metaphase plates of *Festulolium* plants derived from the amphiploid *F. pratensis* (4x) × *L. perenne* (4x) hybrid. Total genomic DNA of *L. perenne* was labelled with FITC and used as a probe (*yellow color*); genomic DNA of *F. pratensis* was used as blocking DNA. Chromosomes were counterstained using propidium iodide (*red color*). **a** F_1 plant (14 *Lolium* + 14 *Festuca* chromosomes). **b** F_6 plant (19 *Lolium* + 9 *Festuca*, including recombinant chromosomes) (Photo Z. Zwierzykowski)

change over generations (Zwierzykowski et al. 1998, 2006; Canter et al. 1999; Kopecký et al. 2006) with impact on value for cultivation and use (VCU) and fertility.

2.2 Introgression

In contrast to the amphiploidy strategy, polyploid hybrids are only transient bridge species and their fertility/stability is not such a crucial factor, provided that there is sufficient fertility in the F1 hybrids to facilitate the transfer of donor genes for the selected trait into the recipient species during a backcross breeding program. Using the hybrid and its derivatives as male parent over one or two backcross generations, the genome of the recipient species is largely restored with little or no compromise either to its phenotype or to its fertility (Morgan et al. 1988; Humphreys 1989; Zwierzykowski et al. 1999; Humphreys et al. 2005).

The efficiency of the introgression breeding approach relies on relatively high frequencies of homeologous chromosome pairing in order to encourage genome recombination. Among all crop plants the *Lolium–Festuca* complex offers the greatest opportunity for homoeologous chromosome pairing in hybrid combinations (King et al. 1999; Zwierzykowski et al. 2008) making introgression a promising breeding strategy for future crop improvement initiatives aimed at increasing the sustainability of *Lolium* (Humphreys 1989; Humphreys and Ghesquière 1994).

3 *Festuca* × *Lolium* F1 Hybrid Production

Festuca × *Lolium* F1 hybrids are relatively easy to produce by emasculating either parent species as female, with *Lolium* used most frequently, and in this case, bagging them with inflorescences of a *Festuca* pollinator species, followed by *in vitro* rescue of hybrid embryos 10–16 days after pollination. Arakawa et al. (2004) reported attempts to produce F1 hybrids at a large scale by using cytoplasmic male sterility found in Italian ryegrass (Komatsu 1987). Somatic fusion of protoplasts was also developed to enhance high rate of interspecific chromosome rearrangements and to produce regenerants of a wide range of ploidy level (Takamizo et al. 1991; Fournier et al. 1996). However, F1 hybrids thus far developed and used for breeding *Festulolium* varieties have been all obtained by sexual reproduction. Although diploid *Lolium* species when intercrossed with a diploid or polyploid *Festuca* species may produce F1 hybrids, these have low female, and very low, or virtually no male fertility, which limits their use in crop improvement programs.

Strategies for restoration of fertility in F1 hybrids comprising entire sets of *Lolium and Festuca* chromosomes have involved polyploidy via application of a chemical such as colchicine that deters spindle construction during cell division. Once F1 amphiploid hybrids are recovered, these are sufficiently fertile to derive next generations by polycrossing under controlled conditions and without

requiring further embryo rescue. This has been demonstrated consistently in crop improvement programs involving hybrids between *Lolium* and either *F. pratensis*, *F. arundinacea*, *F. glaucescens*, *F. gigantea*, *F. mairei,* or *F. arundinacea* var. *atlantigena.*

Amphiploid *L. perenne* or *L. multiflorum* × *F. pratensis* hybrids are also highly amenable to androgenesis. The benefits of this are the identification of gene combinations for extreme variations in the expression of a range of traits such as drought resistance or freezing tolerance (Humphreys et al. 1998; Zare et al. 1999 Leśniewska et al. 2001; Zare et al. 1999) and the capability to fix favorable interspecific gene combinations by subsequent chromosome doubling and restoring disomic chromosome sets.

4 Technical Approaches Used Currently in Breeding *Festulolium*

To date 42 cultivars have been quoted at least once as *Festulolium* in literature (Table 1), of which 24 are registered in the OECD[1] list of 2009 which is required for seed marketing over most countries in the world (http://www.oecd.org/document/14/0,3343,en_2649_33905_41097230_1_1_1_1,00.html). In many cases, the origins of cultivars are poorly referenced. Some cultivars among the very first *Festulolium* are no longer registered on the OECD list (e.g., 'Prior' or 'Elmet'), while *Festulolium* cultivars registered in former eastern or central Europe have been incorporated in the EU list following Germanys reunification and the extension of the European Union to 27 countries.

4.1 Amphiploid Cultivars

Twenty-three *Festulolium* cultivars, all tetraploids (2n = 4x = 28) have been bred using an amphiploidy approach (Figure 2a and c). They derive mostly from reciprocal hybrids of *L. multiflorum* × *F. pratensis. F. pratensis* has been used as female parent on occasions (e.g., cv 'Agula', 'Felopa', 'Sulino') where it was thought cytoplasm inheritance may benefit expression of *Festuca*-specific traits. Casler et al. (2001) reported the registration of cv. 'Spring Green' derived from the intercrossing of four cultivars involving the two types of amphiploid hybrids: 'Elmet', 'Tandem', 'Kemal' (*L. multiflorum* × *F. pratensis*), and 'Prior' (*L. perenne* × *F. pratensis*).

Approaches in development of *Festulolium* hybrids between *Lolium* and *F. arundinacea* have targeted the generation of 8x amphiploids by chromosome doubling 4x hybrids of *L. multiflorum* × *F. arundinacea* (Zwierzykowski 1980) (Figure 2b). However, attempts at stabilizing chromosome number over generations failed to prevent rapid chromosome loss down to around 42, at the expense of chromosomes of *Lolium* origin (Kleijer 1987). However, Pedersen et al. (1990) registered a

[1] Organisation for Economic Co-operation and Development.

Festulolium germplasm 'KY-2N56' with 2n = 8x = 56 chromosomes and claimed it as stable (Eizenga et al. 1991). It is noteworthy that 'KY-2N56' along with the US cultivars 'Kenhy' and 'Johnstone' derived from *L. multiflorum* × *F. arundinacea* hybrids were registered initially in the USA as tall fescue and are not included in the

Table 1 Intergeneric *Lolium* × *Festuca* and *Festuca* × *Lolium* hybrid combinations having resulted in *Festulolium* cultivars following either an amphiploidy (**a**) or an introgression (**b**) breeding approach (in bold cultivars registered on the OECD list in 2009)

(a) Amphiploid *Festulolium* cultivars	Reference
L. multiflorum × *F. pratensis*	
Cultivar	
'**Achilles**'	
'Elmet'	Lewis et al. (1973)
'HŽ14DK'	
'**Emrys**'	
'**Festum**'	
'**Lifema**'	
'**Perseus**'	Houdek (2005)
'**Perun**'	Fojtik (1994)
'Rakopan'	Zwierzykowski et al. (1998)
'**Tatay II**'	
F. pratensis × *L. multiflorum*	
'**Agula**' – '**Felopa**' **–** '**Sulino**'	Zwierzykowski et al. (1998)
'Paulena'	
'**Paulita**'	Netzband (1991)
'**Punia DS**'	Nekrošas et al. (1995)
L. perenne × *F. pratensis*	
'FuRs9806'	Østrem and Larsen (2008)
'Prior'	Lewis et al. (1973)
'Saikava'	Gutmane and Adamovich (2005)
'Spring Green'	Casler et al. (2001)
L. multiflorum × *F. glaucescens*	
'**Lueur**'	Ghesquière et al. (1996)
'**Lusilium**'	
'**Luxane**'	
(b) *Festulolium* cultivars resulting from introgression	Reference
L. multiflorum × *F. pratensis*	
'Evergreen' – 'Kemal' – 'Tandem'	
L. perenne × *F. pratensis*	
'Barfest' – 'Duo' – 'Matrix'	
L. multiflorum × *F. arundinacea*	
'**Bečva**' – '**Lofa**' – '**Fojtan**'	Fojtik (1994)
'Puga'– 'Vėtra'	Nekrošas et al. (2007)
'Felovia'	Boller (pers. comm.)

Table 1 (continued)

(b) *Festulolium* cultivars resulting from introgression	Reference
L. multiflorum × ***F. arundinacea***	
'**Felina**' – '**Hykor**' – '**Korina**' -'**Lesana**'	Fojtik (1994)
'Kenhy'	Buckner et al. (1977)
'Johnstone'	Buckner et al. (1983)
'KY2N56'	Pedersen et al. (1990)

– In bold are receptor species of *Festulolium* cultivars resulting from introgression;
– amphiploid *Festulolium* cultivars are as well as those coming from introgression into Lolium spp. are all tetraploid (2n = 4x = 28) with the exception of cultivar 'Matrix' which is diploid (2n = 2x = 14);
– Cultivars coming from introgression into *F. arundinacea* are all hexaploid (2n = 6x = 42) but cultivar 'KY2N56' which is octoploid (2n = 8x = 56), Eizenga et al. (1991); note that cultivars 'Kenhy,' 'Johnstone,' and 'KY2N56' derive from initial 8x amphiploid hybrids without any backcross into tall fescue;
– The cultivar 'Spring Green' was developed from the intercrossing of four cultivars: 'Elmet' (18%), 'Tandem' (17%), 'Kemal' (50%), and 'Prior' (15%).

present OECD *Festulolium* list. The genome composition of these 6x cultivars is not documented but likely, it should not differ much from cultivars such as 'Hykor' and 'Felina' derived after backcrossing into tall fescue, so that they should be considered more as introgressive forms than true *Lolium* × *Festuca* amphiploids.

From *F. glaucescens*, only 4x amphiploids with *L. multiflorum* (Figure 2c) have been released as *Festulolium* cultivars (Ghesquière et al. 1993, 1996) in the French list. Although 4x F1 hybrids from *L. perenne* have also been successfully produced in the past, they were of insufficient fertility to be bred as cultivars. Alternatively, 4x hybrid ryegrass (*L. multiflorum* × *L. perenne*) can be used in crosses with *F. glaucescens* for combining *Festuca* traits specifically with perennial ryegrass. 6x amphiploids *L. multiflorum* × *F. glaucescens* were only obtained successfully following chromosome doubling of the parental species. Although these 6x amphiploids were phenotypically similar to 4x F1 hybrids and are of potential agronomical value (Ghesquière et al. 1994), their chromosome number was quite unstable over generations similar to the 8x amphiploids developed from *F. arundinacea*.

4.2 Introgression Cultivars

Introgression has been investigated at all ploidy levels into all parental species with the exception of *F. glaucescens*, from 3x, 4x, or 5x F1 hybrids. Nineteen cultivars have been developed following this approach including the six cultivars 'Evergreen,' 'Duo,' 'Tandem,' 'Barfest,' 'Kemal,' and 'Matrix' with no publication reported whether they are actually derived from backcrossing. All are tetraploid except cv.

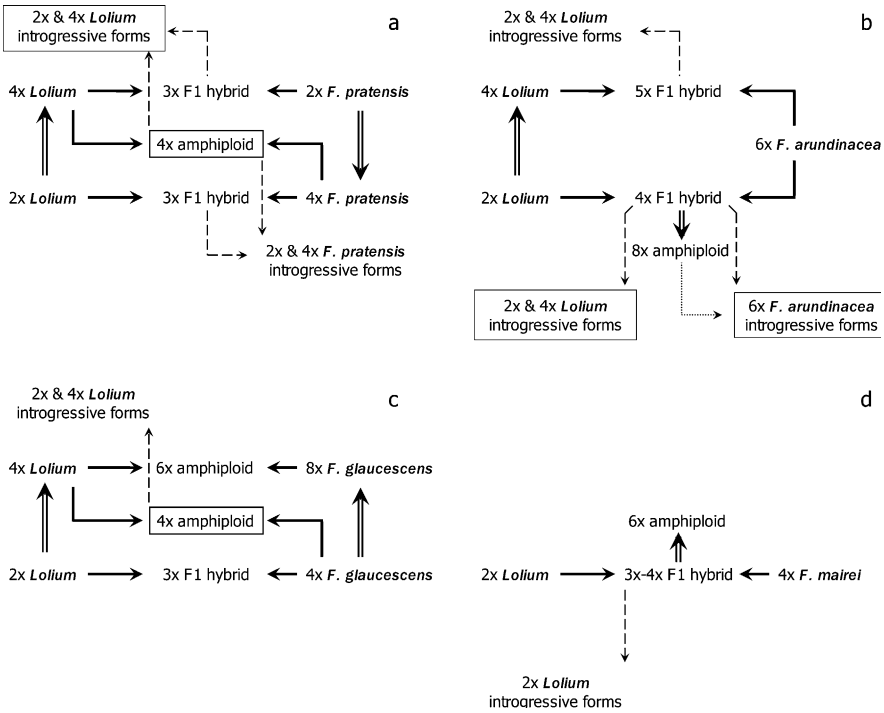

Fig. 2 Main crossing schemes for deriving amphiploid or introgressive *Festulolium* breeding populations using *F. pratensis* (**a**), *F. arundinacea* (**b**), *F. glaucescens* (**c**), or *F. mairei* (**d**) as progenitor. Genome origin and structure of present *Festulolium* cultivars are framed. *Solid arrow*: pair-cross; *double arrow*: colchicine-induced chromosome doubling; *dashed arrow*: backcross; *dotted arrow*: loss of *Lolium* chromosomes and drift to $2n = 6x = 42$

'Matrix'. Those cultivars were classified as introgression on the basis that they phenotypically appeared quite *Lolium*-like. Kopecký et al. (2006) were unable to detect the presence of any *Festuca* chromatin introgression in cultivars 'Duo,' 'Kemal,' and 'Matrix'. However, Yonemaru et al. (2004) found that the rate of natural fluorescence of root tips (a highly *Lolium*-specific phenotypic trait) ranged from 34 to 50% within these cultivars while a *F. pratensis*-specific SSR marker was also evidenced in some of them by Momotaz et al. (2004).

Tetraploid F1 hybrids between 2x *L. multiflorum* and 6x *F. arundinacea* were extensively used by Fojtik (1994) for introgression into both parent species. Four cultivars were released following introgression into tetraploid *L. multiflorum*. However, again no *Festuca* chromatin was detected by GISH in cultivars 'Lofa,' and 'Bečva,' which consistently display a pronounced *Lolium*-like aspect for most traits.

In the converse approach, i.e., introgression of *Lolium* into *F. arundinacea,* four cultivars 'Hykor,' 'Felina,' 'Lesana,' and 'Korina' were bred in the Czech Republic (Fojtik 1994). In this case, maintenance of the introgressed *Lolium* genome was found to be much higher with up to 7.5 intact *Lolium* chromosomes (cv. 'Lesana')

or to 10.21 translocated chromosomes (cv. 'Hykor') (Kopecký et al. 2006). Using double probing GISH, it was further confirmed that interspecific recombination between *L. multiflorum* and *F. arundinacea* genomes involved more frequently the *F. pratensis*-related genome than those of *F. glaucescens* as reported previously (Humphreys and Ghesquière 1994).

Although not having yet reached cultivar release, introgression from *F. glaucescens* into 4x *L. multiflorum* (Ghesquière et al. 2000) as well as into 2x *L. multiflorum* (Humphreys et al. 2005) has been also widely investigated, and improved drought resistance was confirmed. This has been undertaken in the same way as from *F. pratensis* but using 4x amphiploids rather than 3x F1 hybrids. Fertility in 3x F1 hybrids (*L. multiflorum* × *F. glaucescens*) was generally very low in agreement with the expectations that *F. glaucescens* is an allotetraploid (Cao et al. 1994). However, fertility is subsequently improved in triploid hybrids formed following a backcross between the 4x amphiploid and 2x *Lolium* as the BC1 hybrids now contain a homologous set of *Lolium* chromosomes, the prerequisite for fertility together with a *F. glaucescens* genome. It is likely for the same reason that androgenesis of 4x amphiploid hybrids derived from *F. glaucescens* produces only deeply sterile dihaploid regenerants.

Recently, a fourth *Festuca* sp. (*F. mairei*) has been included in *Festulolium* introgression breeding aiming at transfer of xerophytic adaptation traits of *F. mairei* into turf-type cultivars of *L. perenne* from 3x *L. perenne*× *F. mairei* F1 hybrids (e.g., Chen et al. 1995). Tetraploid F1 hybrids were also reported as to result from fertilization by unreduced gametes of the *Lolium* parent as well as recovery of 6x amphiploids (Wang et al. 2009), (Figure 2d).

5 Varietal Groups Among Present Cultivars of *Festulolium*

The genome classification of almost all current *Festulolium* cultivars by Kopecký et al. (2006) gave an evolutionary insight of *Festulolium* genetics (Figure 3). It clearly appears that no present *Festulolium* cultivar achieves a perfectly balanced genome composition. All 6x *Festulolium* cultivars have intact *Festuca* chromosomes in excess while 4x *Festulolium* display predominantly translocated chromosomes mixed with intact *Lolium* chromosomes. Versatility of present *Festulolium* cultivars also gives evidence for the role of the *Festuca* species used initially as parent and of the number of generations by which *Festulolium* cultivars are away from the primary F1 hybrids. The most genome-balanced *Festulolium* and also the most stable over generations, most closely resembling the F1 hybrid, is the only available *Festulolium* cultivar derived from *F. glaucescens*, 'Lusilium'. On the other hand, the two 4x *Festulolium* combinations produced from *F. pratensis* with either *L. multiflorum* or *L. perenne* indicate a significant and progressive *Festuca* chromosome loss (Zwierzykowski et al. 2006). Following a backcross into 4x *Lolium* spp., loss of *Festuca* chromosomes is even more dramatically enhanced, so that eventually no

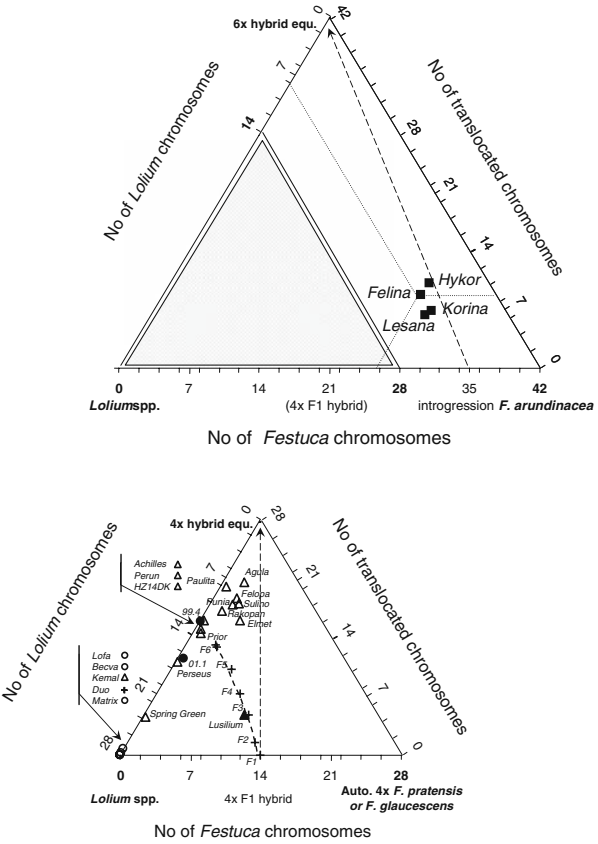

Fig. 3 Genome composition in present *Festulolium* cultivars or experimental breeding populations: 6x *Festulolium* (top) deriving from *L. multiflorum* × *F. arundinacea* (■); 4x *Festulolium* (bottom) deriving from *L. multiflorum* × *F. pratensis* (△), *L. multiflorum* × *F. glaucescens* (▲), or *L. perenne* × *F. pratensis* (+). Introgression into 4x-*Lolium* results from *F. glaucescens* (●) or from *F. arundinacea* or *F. pratensis* (○), except cv. 'Matrix' which is diploid (adapted from Kopecký et al. 2006). F1 to F6 are successive generations of a 4x *F. pratensis* × *L. perenne* population (adapted from Zwierzykowski et al. 2006). *Dotted arrows* indicate expected evolution to equilibrium of chromosome composition in a theoretical 4x hybrid or 6x introgression population of polysomic inheritance under no selection

Festuca genome is evidenced as if introgression has been performed into diploid *Lolium*.

Genome composition in *Festulolium* is likely associated with variability of fertility within cultivar and selection mechanisms over the generations of seed multiplication. The drift to *Lolium* genome could be also linked to the fact that *Festulolium* hybrids having a disomic *Lolium* genome complement are generally more fertile than those having an alternative disomic *Festuca* complement. Recent findings (Humphreys and Zwierzykowski, unpublished data) based on the use of

populations derived in Poland from dihaploid *F. pratensis/L. multiflorum* genomes produced by androgenesis, demonstrate clear evidence for a meiotic drive that favored transmission of *Lolium* compared to *Festuca* chromosomes.

From an end-user viewpoint the current practice for all available *Festulolium* cultivars to all share the same definition while ranging widely in their genome composition and overall VCU may be highly confusing. In the future, registration of *Festulolium* cultivars onto national lists should discriminate between 'Festucoid' and 'Lolioid' types as initially suggested by Fojtik (1994) by gathering on the one hand, all 6x *Festulolium* cultivars which necessarily involve a high genome contribution of tall fescue and, on the other, all 4x and 2x *Festulolium* cultivars resulting from introgression into *Lolium* spp.

6 Major Breeding Achievements

Primary breeding works on *Festulolium* were focused on quality traits within the hexaploid plant material initially developed by interspecific hybridization with tall fescue (e.g., 'Kenhy'). Derivatives were generally more palatable and digestible than tall fescue with large prospects of improvement by subsequent breeding (Buckner et al. 1979). However, insufficient seed production and practically no marketing of these early *Festulolium* cultivars derived from tall fescue prevented any effective measure of their value in respect to quality. When *Festulolium* cultivars were further diversified including amphiploids from *F. pratensis* and introgressive forms into *Lolium* spp. – at least presumed ones – chemical traits linked to digestibility clearly discriminated between them according to parental species and crossing schemes, e.g., cv. 'Felina' against cv. 'Paulita' and 'Evergreen' (Touno et al. 2006). Under low-input systems of autumn-saved herbage for winter grazing, Opitz and Banzhaf (2006) found also that *Festulolium* cultivars resulting from introgression into tall fescue (e.g., cvs 'Felina' and 'Hykor') ranked higher than cv. 'Perun' and 'Lofa,' highlighting the major role of better persistency in the former cultivars due to better winter hardiness and tolerance to fungal diseases.

Likely amphiploid *Festulolium* have given better opportunities for combining overall quality and yield through improved persistency and climatic stress tolerance. Thus, tetraploid *Festulolium* cultivars (*Lolium* spp. × *F. pratensis*) combine the rapid establishment and growth traits of *Lolium* spp. to give high yield in spring with the excellent resilience and winter hardiness of *F. pratensis*. The overall comparison carried out in the SAGES project of the 5th PCRDT of the EU illustrates possibly the best achievements in this respect (SAGES 2004). Various 4x *Festulolium* combinations were compared 2 years long in seven locations across Norway, France, Poland, and Wales against the best local control cultivars of pure species and of the same flowering date (Figure 4). It was obvious that the stress tolerance ranking of *Festulolium* cultivars relative to their parental species depended closely on the location of the field trial. The continental climate of Poland emphasized the winter tolerance of *F. pratensis* and to a lesser extent of *F. arundinacea* while

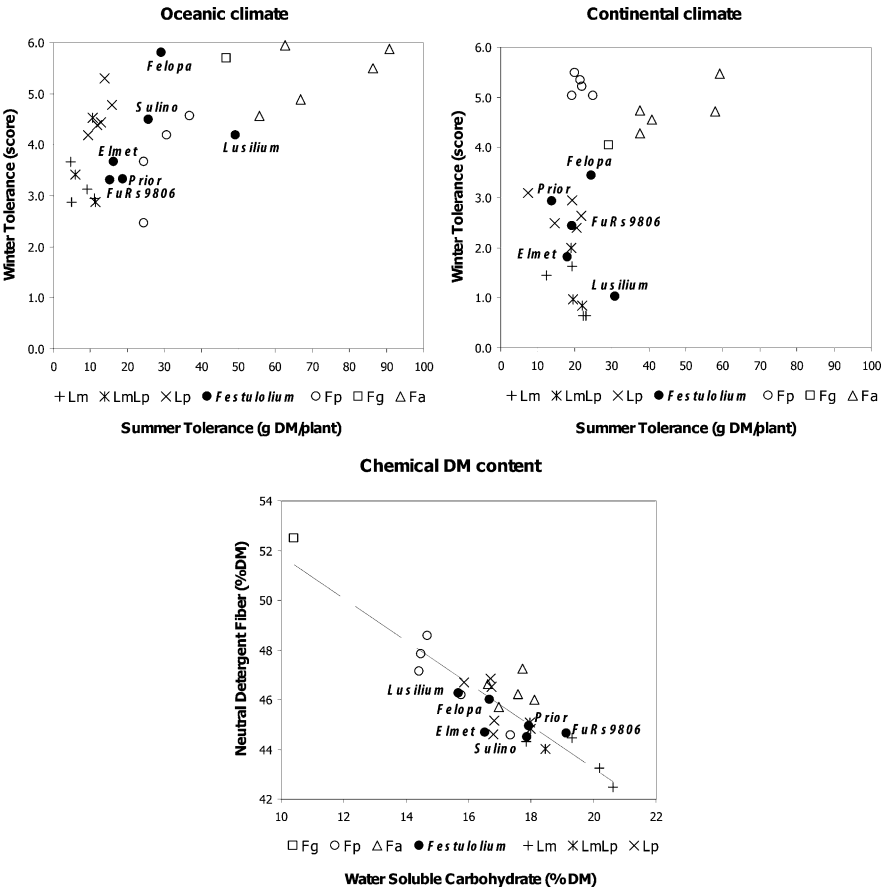

Fig. 4 Chemical composition and response to abiotic stress of various *Festulolium* combinations assessed under oceanic and continental climate together with *Lolium* spp. (Lm, Lp), *Festuca* spp. (Fg, Fp, Fa), and *L. multiflorum* × *L. perenne* (LmLp) control cultivars. *Festulolium* cultivars 'Felopa,' 'Sulino,' and 'Elmet' derive from *L. multiflorum* × *F. pratensis*, cv. 'Lusilium' – from *L. multiflorum* × *F. glaucescens*, and cultivars 'Prior' and 'FuRs9806' – from *L. perenne* × *F. pratensis.* Means of 20 individuals/cultivar in four blocks randomly designed at each location; oceanic: Lusignan (France) 46°26'N 00°06'E; continental: Szelejewo (Poland) 51°51'N 17°10'E. Chemical compositions are means over seven locations (adapted from SAGES, 2004)

F. glaucescens, although it derives from mountain areas, did not appear so well adapted. *Festulolium* involving *F. pratensis* were found to perform better than alternative *Festulolium* hybrids containing *F. glaucescens,* closely to the best *L. perenne* varieties in some instances. In field trials under south oceanic climates that required an enhanced summer drought tolerance, the best performers were *F. arundinacea* and its related progenitor species *F. glaucescens* and *Festulolium* cv. 'Lusilium' (*L. multiflorum* × *F. glaucescens)*. Official trials for registration of 'Lusilium' in the French national list confirmed that it has improved persistency and yield compared

to Italian and hybrid ryegrass, especially when assessed in dry sites of southern France. In Norway, Østrem and Larsen (2008) reported that *Festulolium* derived from *F. arundinacea* (e.g., cultivars 'Hykor' and 'Fojtan') were more winter tolerant that those derived from *F. pratensis* (e.g., 'Felopa' and 'Perun') while, in Japan, the *Festulolium* cv. 'Felina' was found to be also the most summer tolerant among various *Festulolium* derived from *F. pratensis* (Ushiyama et al. 2004). This suggests that *Festulolium* derived from *F. arundinacea* could be of wider range of climatic adaptation because they accumulate sources of abiotic stress tolerance from both *F. pratensis* and *F. glaucescens* genomes. However, as shown by Kopecký et al. (2006), *Festulolium* derived from *F. arundinacea* also have an overall *Festuca* genome contribution much higher than those derived from *F. pratensis*.

As far as chemical composition is concerned, neutral detergent fibber (NDF) and water soluble carbohydrate (WSC) content also significantly discriminated between ryegrass and fescue controls over all locations in the SAGES project (Figure 4). However, chemical composition among the amphiploid *Festulolium* against parental species was inconsistent due to large genetic variability within species. In absolute values, NDF and WSC content in both *Festulolium* derived from *L. perenne* × *F. pratensis* (cv 'Prior' and 'FuRs9806') reached that of the best *L. multiflorum* cultivars. Among the fescues, *F. glaucescens* had extreme NDF and WSC content; yet, the *Festulolium* cv. 'Lusilium' derived from *L. multiflorum* × *F. glaucescens* had a level of chemical composition only a little less than that of the five *F. pratensis* × *L. perenne/multiflorum* -derived *Festulolium* cultivars (Figure 4). Early assessment of digestibility in the amphiploid population having led to cv. 'Lusilium' showed that it was at an intermediate level between tall fescue and Italian ryegrass with significant responses to selection using palatability test with animals (Ghesquière et al. 1996).

Disease tolerance gained in *Festulolium* breeding is poorly reported because most approaches involved the use of intraspecific genetic variability from parental sources. In cases where disease tolerance in parent genitors was not sufficiently assessed before hybridization, generally the resulting *Festulolium* populations may not have met standard VCU requirements; Suter et al. (2007) reported that five *L. multiflorum* × *F. pratensis* cultivars failed for registration in the recommended list of Switzerland because of susceptibility to bacterial wilt (*Xanthomonas campestris*), likely originating from *L. multiflorum* parent. It is noteworthy that winter tolerance sought in *Festulolium* may emphasize new specific requirements such as tolerances to snow mould (*Microdochium nivale*) if long periods of snow cover are foreseen. Generally, disease tolerance is higher in *Festuca* spp. which explains that introgression into *Lolium* spp. rather than an amphiploidy approach is carried out for breeding more tolerant *Festulolium* cultivars (e.g., Adomako et al. 1997) with the prime example being the transfer of crown rust (*Puccinia coronata*) resistance from *F. arundinacea* and *F. pratensis* into *L. multiflorum* (Oertel and Matzk 1999) and from *F. pratensis* into *L. perenne* chromosome 5 (Roderick et al. 2003).

In conclusion, rather than expecting better performances of single traits relative to their parental species, the benefit of tetraploid *Festulolium* would be

better acknowledged by its potential to achieve an optimum balance in productivity, quality, and stress tolerance that is likely to be unreachable by intraspecific conventional breeding. Touno et al. (2006) showed that *Festulolium* cv. 'Evergreen' combined feeding and winter tolerance traits in northeastern Japan at a level capable of rivalling the *Dactylis glomerata* control. Interestingly, genetic variability within *Festulolium* cultivars grown in stands can also give rise to adaptive response for freezing tolerance (Casler et al. 2002; Yonemaru et al. 2004) and for persistency in mixtures with legumes. This suggests that genetic changes in *Festulolium* could not be only genome driven but also result from differential fitness of phenotypic traits in strong linkage disequilibrium between parent species. In this respect, present *Festulolium* cultivars are obviously as much the result of breeding as that of antagonistic selective forces at genome and phenotype level.

7 Seed Production

Fertility and seed productivity are crucial aspects of breeding *Festulolium*. Research generally concentrated on the early generations following primary interspecific hybridization by assessing male and female components of fertility: anther dehiscence, pollen stainability, seed set, and regularity of chromosome pairing at meiosis. Using selection for fertility in 4x *L. multiflorum* × *F. pratensis* hybrids, seed set under open pollination increased from 28.4% in F2 to 61.0% in F4, and stabilized in the next two generations at about 55.0% (Zwierzykowski et al. 1993), which approaches the level of 55–65% commonly found in diploid ryegrass and meadow fescue varieties. In 4x *L. multiflorum* × *F. glaucescens* hybrids, fertility was found to have been improved significantly from 1.19 seed/spikelet in F1 to 1.82 in F2 generation while stabilized at 1.95 in F3 (Ghesquière et al. 1993).

When breeding programs are more advanced, selection for improving seed yield can be based on total seed weight per plant or average seed weight per head among genotypes entering in polycross. Although large genetic variability and high heritability were generally found in *F. glaucescens*-derived hybrid populations, correlation of seed yield between mother plant and HS progeny remained quite low with generally no response over generations of selection (Ghesquière and Bourgoin 2009).

Seed yield potential is generally well recovered in *Festulolium* resulting from introgression, close to the pure parent species, especially into 4x *Lolium* spp. However, when introgression actually resulted from hybridization with tall fescue, seed yield always remained low and not liable to improvement despite genetic variability was found (Burner et al. 1991). This probably explains that cultivars such as 'Kenhy' or 'Johnstone' were so little marketed following early release in the USA in the 1980s. Possibly doubling the chromosome number of primary 4x hybrids into 8x amphiploids adds to the intrinsic detrimental effect of *F. glaucescens* genome on fertility of so-derived *Festulolium*. However, when resulting from backcrossing into tall fescue, *Festulolium* cultivars like 'Hykor' and 'Felina' appear to have a more acceptable seed yield.

Table 2 World seed production area in 2007 and average seed yield reported from present *Festulolium* cultivars

Initial hybrid combination and cultivar name (country of origin)	Area (ha)	Seed yield (dt/ha) Plot trials			Multiplication fields		
L. multiflorum ×*F. pratensis*							
'Lifema' (GER)	–	–	6.3[d]	–	–		
'Achilles' (CZ)	60[a]	–	–	–	6.0[a]		
'Perun' (CZ)	795[a]	6.28[c]	–	–	6.0[a]		
'Perseus' (CZ)	90[a]	–	–	–	6.0[a]		
F. pratensis × *L. multiflorum*							
'Felopa' (POL)	762[b]	–	7.6[d]	5.2 – 7.0[e]	–		
'Sulino' (POL)	294[b]	–	–	5.2 – 7.0[e]	–		
'Agula' (POL)	154[b]	–	–	5.2 – 7.0[e]	–		
'Paulita' (GER)	–	–	10.5[d]	–	–	6.0[f]	5.0[g]
L. multiflorum × *F. glaucescens*							
'Lueur' (FRA)	–	–	8.1[d]	–	–		
'Lusilium' (FRA)	–	–	5.5[d]	–	–		
'Luxane' (FRA)	–	–	5.7[d]	–	–		
L. perenne × *F. pratensis*							
'Duo' (USA)	–	–	7.1[d]	–	–		

Table 2 (continued)

Initial hybrid combination and cultivar name (country of origin)	Area (ha)	Seed yield (dt/ha) Plot trials			Multiplication fields
L. multiflorum $\times$ *F. arundinacea*					
'Lofa' (CZ)	570[a]	–	11.9[d]	–	10.0[a]
'Bečva' (CZ)	55[a]	12.4[c]	–	–	8.0[a]
'Fojtan' (CZ)	100[a]	–	–	–	4.0[a]
L. multiflorum $\times$ ***F. arundinacea***					
'Felina' (CZ)	190[a]	8.0[c]	–	–	4.0[a]
'Hykor' (CZ)	520[a]	9.9[c]	–	–	4.0[a]
'Korina' (CZ)	25[a]	–	–	–	–

In **bold** are receptor species of *Festulolium* cultivars resulting from introgression, amphiploid cultivars otherwise.
[a]V. Černoch (pers. comm.)
[b]W. Jokś (pers. comm.).
[c]Average of 6 trials from 1987 to 1992 (Fojtik 1994).
[d]Ghesquière and Bourgoin (2009).
[e]Jokś et al. (1994)
[f]Netzband (1991)
[g]Bundessortenamt (2001).

Real seed yield assessment through comparative trials in plots is very little documented. In Table 2, various sources show that seed yield may considerably vary among *Festulolium*. Cultivars like 'Bečva,' 'Lofa,' and 'Paulita' appear to be the most steadily highly productive. However, seed yield of *Festulolium* cultivars in trials may be overestimated due to better pollen availability and higher rates of fertilization than in practical seed production. At the scale of multiplication fields, seed yield is often much less as seen from cv. 'Paulita,' while cv. 'Lofa' confirms its high yielding potential. As a matter of fact, the actual seed yield of *Festulolium* cultivars relatively to pure parent species remains largely unknown unless subsequent seed production and marketing can return data. *Festulolium* introgression cultivars 'Hykor' and 'Lofa' were the most seed multiplied over the world in 2007, followed by amphiploid cultivars 'Felopa' and 'Perun' (Table 2). Similarly, cv. 'Lofa' was by far the most seed multiplied and marketed in France in 2007 by about 350 ha, although the *Festulolium* seed market overall steadily decreased since 2004.

The best seed yielding *Festulolium* cultivars come from introgression into *Lolium*; they are also those in which no *Festuca* chromatin was found using GISH technique. This suggests that seed production potential may negatively correlate among cultivars with overall hybridity and hence, agronomical performances for interspecific traits. It is not known yet whether the relation could be close enough to prevent any further joint progress. To find the right genome balance between *Lolium* and *Festuca* will be definitely the challenge for future breeding of polyploid *Festulolium*.

8 Integration of New Biotechnologies in Breeding Programs and Prospects

All *Festuca* species have been employed thus far primarily as sources of genes for drought and cold resistance by incorporating the introgression strategy developed in the UK (Morgan et al. 1988; Humphreys 1989). Other traits include crown rust resistance and delayed senescence. (Table 3). In the EU funded project SAGES (SAGES 2004), introgression of *Festuca* chromosomes 3 and 5 were found to correlate with enhanced drought resistance in the EU funded project SAGES (SAGES 2004) with consistent marker associations on chromosome 3F of *F. pratensis* with QTL (*QDts3F*) for growth under severe drought stress along its entire length. No similar QTL for resistance to drought stress has been observed in *Lolium* (Turner et al. 2008) indicating the value of this *Festuca* chromosome as a source of novel variation with benefit to *Lolium.* Three independent alien introgressions derived from chromosome 3 from either *F. arundinacea* or *F. glaucescens* (Humphreys et al. 2005) are being employed currently in the UK crop improvement programs. This chromosome is orthologous to rice chromosome 1 and is known to have QTL associations with osmotic adjustment, dehydration tolerance, and numerous rooting characters.

Ghesquière et al. in the SAGES program identified and transferred into *Lolium*, under a *F. glaucescens*-derived genes for drought resistance from *Festuca*

Table 3 Introgression of traits from *Festuca* spp. into *Lolium* spp.

Introgressed trait	Donor species	Recipient species	Reference
Drought resistance	*F. arundinacea*	*L. multiflorum*	Humphreys and Thomas (1993) Humphreys and Ghesquière (1994) Humphreys and Pašakinskienė (1996)
	F. glaucescens	*L. multiflorum*	SAGES (2004) Humphreys et al. (2005)
	F. mairei	*L. perenne*	Chen et al. (1995)
Drought and cold tolerance	*F. arundinacea*	*L. multiflorum*	Humphreys et al. (1997) Skibińska et al. (2002) Kosmala et al. (2003)
	F. pratensis	*L. multiflorum*	Skibińska et al. (2002) Kosmala et al. (2003)
Freezing tolerance	*F. pratensis*	*L. multiflorum*	Kosmala et al. (2006)
	F. pratensis	*L. perenne*	Grønnerød et al. (2004)
Winter hardiness and freezing tolerance	*F. arundinacea*	*L. multiflorum*	Kosmala et al. (2007)
Crown rust resistance	*F. pratensis*	*L. multiflorum*	Oertel and Matzk (1999) Roderick et al. (2003)
	F. pratensis	*L. perenne*	Adomako et al. (1997)
	F. arundinacea	*L. multiflorum*	Oertel and Matzk (1999)
Sid – delayed senescence	*F. pratensis*	*L. multiflorum*	Thomas et al. (1997)
	F. pratensis	*L. perenne*	Thomas et al. (1997)

chromosome 5 (SAGES 2004). Durand et al. (2007) demonstrated how *L. multiflorum* × *F. glaucescens* hybrids due to their enhanced rooting capabilities are able to extract water from greater soil depths than the ryegrass parent when plants are exposed to severe drought stress. Better root penetration of hard compacted soils will also increase grass forage yields in addition to the benefits for improved drought resistance. Improved root design can stabilize soils thereby preventing erosion. There are large differences in soil hydraulic conductivity depending on the species and cultivars grown. For example, macro-porosity in soils supporting cultivars of *F. arundinacea* was markedly greater when compared to those under *L. multiflorum*. In 2008 over 30 rainfall episodes in the UK, run off of water at different soil depths was 50% lower in soils under a *L. perenne* × *F. pratensis* cultivar than under a *L. perenne* cultivar and 30% lower than under a *F. pratensis* cultivar (MacLeod et al. (unpublished)) opening new opportunities for

Festulolium as safeguards against flooding following extreme rainfall, a scenario expected to increase in frequency and intensity with climate change.

Introgression of winter hardiness into *Lolium* spp. involved gene transfers from *Festuca* into *Lolium* chromosome 3 (Grønnerød et al. 2004), chromosome 2 (Kosmala et al. 2006, 2007), and chromosome 4 (Humphreys et al. 2006) each leading to significant improvements to *Lolium* in freezing tolerance. The findings from the British–Polish collaboration involving the gene transfer from *F. pratensis* chromosome 4 were particularly valuable. In *Lolium* and *Festuca* species, new evidence of the importance of the adaptive capabilities of Photosystem II (PSII) during cold acclimation in relation to subsequent freezing tolerance was demonstrated. Non-photochemical quenching (NPQ) mechanisms for expulsion of excess light energy were found in *F. pratensis,* but not to the same extent in *L. multiflorum.* A direct relationship between cold acclimation, increases in NPQ and freezing tolerance were reported. Evidence of a role for genes found on chromosome 4 of *F. pratensis* for increased NPQ expression was found and later confirmed and localized using a *F. pratensis* chromosome substitution series developed at IBERS (Humphreys and Gasior, unpublished data).

Without question, the advances in development of new gene marker technologies and several detailed QTL linkage studies have provided opportunities for precision breeding and assembly of key alleles for desirable complex agronomic traits to be employed in present and future *Festulolium* breeding programs. The marker systems have developed from early research using isozymes (e.g., Humphreys and Ghesquière 1994), through use of AFLPs and STS markers (e.g., Humphreys et al. 2005), to microsatellites (e.g., Momotaz et al.2004), to SNP development, and recently the employment of DArT markers within the *Lolium–Festuca* complex (Kopecký et al. 2009).

Introgression-mapping (Humphreys et al. 1997; King et al. 2007) will allow the 'dissection' of the genes controlling the major traits and facilitate the application of appropriate gene markers to the selection of major genes and facilitate their employment in crop improvement programs. The generation of complete sets of chromosome substitution lines from *Lolium/Festuca* hybrids now exists both in diploid populations (King et al. 2002) and in tetraploids (Kopecký et al. 2008) and will assist greatly in genomic and phenomic screens to identify key alleles and for map-based cloning and marker-assisted gene transfer for plant breeding.

9 Conclusions

Festulolium cultivars provide specialist function and novel alternatives to existing grass cultivars that may either lack the quality of *Festulolium* or their resilience against abiotic or biotic stresses. They may be viewed as possible alternatives to the use of seed mixtures, or for a specialist use. In the longer term, should our climates become consistently warmer and drier during the summer and/or liable to flooding

due to extreme incidents of rainfall during autumn and winter, then their use may well increase.

Although dispersed throughout the world, *Festulolium* breeding has considerably stimulated research on genetics of the grasses and has contributed to the development of new technologies. Obviously, there is a gap between *Festulolium* breeding, which is currently applied on a plant material of essentially polyploid nature and future precision breeding aimed at the transfer and introgression of selected genes into diploid *Lolium* spp. However, provided that regulation for registration in national lists still allows acknowledgment of the originality and agronomic advances of future *Festulolium* cultivars, it would seem very likely that polyploid *Festulolium* could play a role for a better understanding of genome evolution in the grasses. In this respect, it is not unrealistic that breeding polyploid *Festulolium* could also benefit from the genomic advances achieved in diploids particularly if stress tolerance is genetically controlled by many co-adapted genes, physically and functionally organized at the scale of the chromosomes.

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Cocksfoot

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1 Introduction

Cocksfoot (orchardgrass, *Dactylis glomerata* L.) is a long-lived perennial grass, well adapted to temperate zones of the world. It has good regrowth characteristics and adaptability to various environmental conditions. Cocksfoot is widely distributed in most European countries, North America such as the USA and Canada, South America, Australia, New Zealand, and Asia. It shows heat and drought tolerance under Mediterranean climatic conditions but is not tolerant to water logging and wet soils and only moderately winter hardy. Cocksfoot is used for hay, silage, and grazing, and is suitable for mixed sowing with alfalfa (*Medicago sativa* L.) or red clover (*Trifolium pratense* L.) for hay or white clover (*Trifolium repens* L.) for grazing. The main advantage of this species is greater forage production during summer compared to other forage grasses; however, its forage quality is lower than that of the highly digestible grasses such as *Lolium* spp. Because of its shade tolerance cocksfoot is also used in Europe to establish vegetation covers in vineyards or orchards.

Breeding programs of cocksfoot in continental Europe and Great Britain (Beddows 1968), Canada and the USA (Lawrence et al. 1995; van Santen and Sleper 1996; Casler et al. 2000) began around the 1930s. Cocksfoot breeding has been conducted by the public sector mainly in the USA, Canada, Japan, Australia, and New Zealand, and in Europe mainly by private section entities since then. Public cocksfoot breeding in France began in 1953 at INRA Versailles and was continued in Lusignan from 1962. First private companies began their programs in cooperation with the public institutes in the 1970s (Mousset 2000). Most current French cultivars originated from these cooperative ventures. Today all cocksfoot breeding programs in France are private.

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2 Origin and Systematics

Cocksfoot originates from Eurasia and was introduced into the USA in the 1750s (Balasko and Nelson 2003). Seed was shipped back to Great Britain from Virginia, USA in 1763 (Beddows 1968). Settlers from western Europe also introduced the species into South America, Australia, New Zealand in the last three centuries (Lolicato and Rumball 1994). It was introduced into Japan from the USA in the 1870s.

The monospecific genus *Dactylis* L. is a member of the Poeae tribe of the Pooideae subfamily (Watson et al. 1985; Watson et al. 1986) with x = 7 as the basic chromosome number characteristic for this tribe. It consists of the single species *Dactylis glomerata* L. (Domin 1943) and at least 18 subspecies at $2n = 6x = 42$, $2n = 4x = 28$, and $2n = 2x = 14$ ploidy levels. Most subspecies are diploids (Lumaret 1988), but 'tetraploids represent about 95% of the genus, and display great morphological variability' (Lindner et al. 2004). Lumaret (1988) in a comprehensive review provides a map of the distributional ranges for these subspecies, of which 2x species are considered the most ancient. Fiasson et al. (1987) proposed a dual-branched evolutionary tree with a temperate branch derived from ssp. *aschersoniana* and a Mediterranean branch originating from material related to subsp. *aschersoniana*, *smithii*, and *juncinella*. A newly named 2x subspecies from Galicia, Spain – ssp. *izcoi* (Ortiz and Rodriguez-Oubina 1993) – has been the subject of a series of investigations (Lindner et al. 2004). Sahuquillo and Lumaret (1999) used chloroplast RFLPs to demonstrate gene flow from Mediterranean taxa into subtropical material in the Macaronesian islands. Tuna et al. (2004) concluded that 2x species were rare to non-existent in Turkey based on RAPD markers.

Numerous recent studies confirm older evidence that 2x subspecies have very restricted distributional ranges (Amirouche and Misset 2007; Gauthier et al. 1998; Guignard 1985; Jay and Lumaret 1995; Lindner and Garcia 1997). Tetraploids, particularly ssp. *glomerata*, occupy a much wider geographic range. They arose from 2x progenitor through chromosome doubling *sensu latu* (Lumaret 1988 and references therein). Both climatic changes and human activity such as forest clearing contributed to the expansion. Studies also indicate that hybridization between 2x and 4x species occurs both in natural (Jones and Borrill 1962; Lindner et al. 2000; Lumaret and Barrientos 1990; Lumaret and Hanotte 1987; Sahuquillo and Lumaret 1999; Stebbins and Zohary 1959) and artificial settings (De Haan et al. 1992; van Santen and Casler 1986; van Santen et al. 1991).

3 Varietal Groups

A total 194 of cocksfoot cultivars were listed on the OECD list of cultivars eligible for certification in 2008. Most of cultivars in this list belong to 4x *Dactylis glomerata* spp. *glomerata*. The maintainers of 133 cultivars are in Europe; 30 cultivars are from Canada and the USA; 7 from Australia and New Zealand; 8 from South America; 16 from Japan. Cocksfoot cultivars are divided into three maturity groups based

on heading date such as early, medium, and late. The difference in heading date between early and late maturing cultivars ranges from 2 to 3 weeks depending on climatic conditions. All cultivars were developed for forage use. Some cultivars were used for forage and revegetation.

4 Genetic Resources and Utilization

There have been intensive efforts to collect *Dactylis* accessions for 70 years. The largest comprehensive database of *Dactylis* accessions is hosted by NORDGEN on behalf of the European Cooperative Program for Plant Genetic Resources (ECPGR) (http://www.nordgen.org/ecpgr/index.php?scope=ecpgr&app=data_unit&unit=ecpgr_dactylis; verified 31 January, 2009) and contains over 11,000 accessions from all European gene banks. The USDA National Plant Germplasm System (NPGS) contains over 1,400 accessions, of which 550 each trace to Europe and Asia, and little over 200 to Africa (http://www.ars-grin.gov/cgi-bin/npgs/html/tax_search.pl; verified January 31, 2009).

Four recent publications may serve to illustrate that there is no lack of genetic variation. An Iranian study found significant variation among 29 accessions from Iran, Europe, and North America for seed yield component traits (Jafari 2004). A Japanese study revealed significant variation for water-soluble carbohydrates (WSC) in vegetative material (Sanada et al. 2004) and a study in Israel significant molecular variation for drought response in 4x *glomerata* (Trejo-Calzada and O'Connell 2005). Finally, a Chinese study involving North American, Australian, northern European, and Chinese (including a single 2x) accessions, revealed 84% polymorphism based on SRAP markers and a clear separation of material from different continents (Zeng et al. 2008).

Cocksfoot breeding has a long history of diverse germplasm utilization. For example, cv. 'Pennlate' (released 1957) was developed from Swedish and Finnish plant introductions (Casler et al. 2001). Phenotypic selection within a single Spanish accession led to cv. 'Grasslands Excel' (Rumball 1999). 'Prairial' (1957) was released from subsp. *glomerata* plant material originating from low elevation non-Alpine areas in France (Gauthier et al. 1998). The first populations for breeding in France were collected 1968–1971 in northwestern France (mild oceanic climate) and were used for the improvement of disease resistance, and for combining late heading with a good spring growth and a high yield level. After two recurrent breeding cycles, the cultivars 'Lully,' 'Lude,' and 'Lutetia' were released, respectively, in 1977, 1978, and 1978 from this material. Next, natural populations collected in the north of Spain and Portugal (1978–1982) were used in the French breeding program to extend the period of growth in autumn. Generally this Mediterranean material was less variable than that coming from the north of France, and sometimes reduced frost resistance.

The recent Canadian cv. 'Kayak' is a selection from the old cv. 'Chinook' (a naturalized strain from Alberta released in 1977), 'Kay' (a 1977 release based on

a Russian accession), and some material of unknown provenance (Acharya et al. 2007). Winter hardiness among European and Japanese cultivars and ecotypes of cocksfoot varied when tested on Hokkaido, Japan (Nakayama et al. 1997; Sanada et al. 2007b), with germplasm originating from northern Europe and Russia showing better winter hardiness, but poorer plant vigor in summer and autumn. Although ecotypes derived from mountainous areas in southern France showed winter dormancy and lower drought tolerance in South Australia (Knight 1973), the same ecotypes showed less fall dormancy and more tolerance to prolonged snow covers when snow mold was controlled through fungicides than the Japanese cultivars on Hokkaido in Japan (Sanada et al. 2007b).

Four cycles of recurrent selection in an accession from Galicia, Spain resulted in the improved cv. 'Megatas' adapted to Tasmania (Hurst and Hall 2007). 'Currie' was selected in Australia from Algerian materials and 'Kasbah' (ssp. *hispanica*) from materials collected in Morocco (Oram and Lodge 2003). Finally, Harris et al. (2008) report the creation of improved synthetic populations involving material from ssp. *glomerata* and *hispanica* based on intense selection on the North West Slopes of New South Wales and on the Central Highlands of Victoria.

It is clear from this review that cocksfoot breeders use germplasm accessions quite intensively, particularly when broadening the potential area of adaptation and utilization, as is the case in Australia.

5 Major Breeding Achievements

More than 200 cultivars have been bred and released in Europe, Australia, New Zealand, Japan, Canada, and the USA since the 1950s. Increasing forage yield, disease resistance, and tolerance to abiotic stress such as drought or cold have been common breeding objectives for these breeding programs. Winter hardiness of cocksfoot is lower than timothy (*Phleum pratense* L.) and meadow fescue (*Festuca pratensis* Huds.), which are used mainly in more northern regions. Improvement of winter hardiness, increasing resistance to snow mold, and freezing tolerance are therefore the most important objectives for cocksfoot in the northern regions. 'Wasemidori', an 8-clone synthetic cultivar, was developed through natural selection for winter hardiness and snow mold resistance. Forage yield was evaluated through progeny testing in the field and freezing tolerance by an artificial freezing test in the laboratory (Terada et al. 1991). The Canadian cultivar 'Kayak' was bred by synthesizing 23 winter-hardy families selected by an artificial freezing test and natural selection for winter hardiness in the field (Acharya et al. 2007).

Resistance against purple leaf spot caused by *Stagonospora arenaria* Sacc. was improved by three to five cycles of phenotypic recurrent selection (Oberheim et al. 1987; Berg et al. 1992, Berg et al. 1990). Powdery mildew caused by *Erysiphe graminis* DC. f. sp. *dactylidis*, already common in Europe for many years, was first found in Japan in the 1960s. Japanese cultivars, well adapted to the Japanese wet climate, were more susceptible to the disease than European and American

cultivars (Fujimoto et al. 1993). A mildew resistant Japanese cultivar 'Akimidori II' was developed by selection among half-sib families in the field under spaced-plant and sward conditions (Sugita et al. 1995). The most important disease in France is leaf streak caused by *Scolecotrichum graminis* (Raynal et al. 1989). Since the end of the 1970s, there has been continued improvement in streak resistance culminating with the release of 'Lutetia'. Since the late 1990s rust (*Puccinia striiformis* var. *dactylidis* and *Uromyces dactylidis*) resistance level of newly released cultivars has been high. All these improvements were mainly achieved by selection after natural infection in field trials.

6 Specific Goals in Current Breeding

Improving leaf disease resistance aims at reducing adverse effects of the disease on forage quality (Edwards et al. 1981; Isawa 1983). Improvement of resistance to stem rust (*Puccinia graminis*) has been a major objective in many cocksfoot breeding programs in the USA, Europe, and Japan (Alderson and Sharp 1995). Two cycles of phenotypic recurrent selection for resistance to stem rust achieved the same level of resistance as did a single cycle of genotypic selection based on a polycross progeny test (Miller and Carlson 1982). Parental clones of the stem rust resistant cultivar 'Akimidori' were selected by artificial inoculation of 9,000 plants of half-sib families and cultivars with stem rust (Kawabata et al. 1977). Stripe or yellow rust caused by *Puccinia striiformis* var. *striiformis* Westend (synonym *P. striiformis* f. sp. *dactylidis* Tollenaar) invaded New Zealand and North America in the late 1970s to the early 1980s (Latch 1976; Hardison 1984), and Japan in the early 2000s (Sugawara et al. 2006). Resistance to stripe rust is already an important objective for cocksfoot breeding programs in North America and Europe and will soon become a major breeding objective in Japan. Drechslera leaf spot caused by *Drechslera dactylidis* Shoemaker, scald caused by *Rhynchosporium orthosporum* Caldwell, and leaf streak (brown stripe) caused by *Scolecotrichum graminis* Fuckel (syn. *Cercosporidium graminis* (Fuckel) Deighton) are all serious diseases in cocksfoot. Compared to the base population, resistance to Drechslera leaf spot increased with one cycle of half-sib family selection for forage yield in 9 out of 11 selected populations (Casler et al. 2002). Resistance to scald in cocksfoot was increased though one cycle of phenotypic selection by artificial inoculation of seedlings (Sugita et al. 1987). Leaf streak resistance was positively correlated with WSC concentration in cocksfoot (Sanada et al. 2004). Selection for resistance to leaf streak and scald may be an effective way to improve the forage quality of cocksfoot.

Improving forage quality has been a major breeding objective in cocksfoot since the 1960s (van Dijk 1959). The correlation of forage yield with neutral detergent fiber (NDF) concentration was positive and, consequently, with *in vitro* dry matter digestibility (IVDMD) was negative (Stratton et al. 1979) but the situation is not always clear-cut (see Casler et al. 2002). The magnitude of the genetic correlation

coefficient between WSC and forage yield under sward (Sanada et al. 2007a) and spaced-plant conditions (Jafari and Naseri 2007) was low, while a positive correlation of acid detergent fiber (ADF) and NDF concentrations with forage yield was found among half-sib families in cocksfoot (Sanada et al. 2007a). These results suggest that it may be possible to improve the forage quality without decreasing forage yield.

7 Breeding Methods and Specific Techniques

Breeding methods of cocksfoot are similar to other cross-pollinated grasses. Most modern cocksfoot cultivars are synthetics bred by genotypic selection based on a progeny test, or phenotypic selection of clones in the USA, Canada, Europe, and New Zealand (Alderson and Sharp 1995; Casler et al. 2000). Half-sib family selection without within-family selection was effective in increasing forage yield (Casler et al. 2002). 'Akimidori II,' selected through half-sib family selection, had a 6% higher forage yield than the check cultivar when evaluated at six locations in southern Japan for 3 years (Sugita et al. 1995). Multi-location selection for forage traits and seed production was successfully conducted at four locations in the USA using four populations of cocksfoot (Casler et al. 1997a; Barker et al. 1997; Casler et al. 1997b). In this program, two cycles of phenotypic recurrent selection were conducted for each population, using the convergent-divergent (C/D) selection scheme (Lonnquist et al. 1979). The results indicate that multi-location selection for agronomic traits can lead to increased forage yield and seed production across a wide range of environments.

Correlations among forage quality traits at the first cut tend to be influenced by the degree of heading and the number of reproductive stems, which affect IVDMD negatively (Stratton et al. 1979; Saiga 1981). It is therefore suggested to select for forage quality traits at the vegetative growth stage (Saiga 1981).

8 Integration of New Biotechnologies in Breeding Programs

Transgenic plants of cocksfoot were obtained from protoplast by electroporation or polyethylene glycol treatment (Horn et al. 1988), young leaf tissue by microprojectile bombardment (Denchev et al. 1997), and seed-derived callus by Agrobacterium-mediated genetic transformation method (Lee et al. 2006). A highly efficient transformation system for cocksfoot was established using microprojectile bombardment of highly regenerative, green tissues derived from mature seeds of cv. 'Rapido'. (Cho et al. 2001). Heat shock protein (HSP) gene isolated from cocksfoot was transformed into embryogenic callus of cocksfoot by Agrobacterium method (Kim et al. 2008). Based on a leaf disk assessment, this transgenic plant showed heat tolerance at 60° C which was lethal for non-transgenic plants.

Molecular markers have been used to evaluate the genetic diversity of germplasm and breeding material. The genetic diversity of cocksfoot germplasm was evaluated by sequence-related amplified (SRAP) marker (Zeng et al. 2008) and amplified fragment length polymorphism (AFLP) (Peng et al. 2008). A linkage map of cocksfoot based on 606 simple sequence repeats (SSR) markers has been developed in a segregating population of F1 between 'Akimodori II' and 'Loki' (Cai et al. 2008).

There is little evidence in refereed journals on the application of new biotechnologies in actual *Dactylis* breeding programs. Application of modern molecular methods is sometimes said to have a potential application in *Dactylis* (Charmet et al. 1997; Forster et al. 2008). Such technologies require quite an investment and one would not expect *Dactylis* to be the primary beneficiary of such an approach. Cocksfoot is commonly used in pastures in the northern USA and eastern Canada, sometimes in mixture with alfalfa for hay production. It is considered indispensable to complement complex perennial ryegrass-white clover mixtures for drier conditions (Suter et al. 2008). Most cocksfoot, however, is used as forage for beef cattle, replacement heifers, or dry cows, which have lower nutritional requirements than high-producing lactating dairy cattle that are very sensitive to the quality of feed on offer. Along with the generally low profit margin in the seed industry, economic incentives do not currently support the application of expensive approaches for a species such as cocksfoot that offers small economic returns. Species valuable as forage/feed for dairy cattle, such as ryegrasses, alfalfa, and forage maize, will remain the most likely targets for application of modern biotechnologies for the foreseeable future. For a species like cocksfoot, there is little opportunity to recoup development costs and/or to pay licensing fees for technologies developed with public funds.

9 Seed Production

Isolation is necessary for the seed production of cocksfoot cultivar or population to protect from contamination of pollen from other cultivars or feral populations, because cocksfoot is cross- and wind pollinated. The isolation distances in the field must not be less than 200 m in seed production for further multiplication. Polycrossing to produce the seed for progeny test or next cycle of recurrent selection is usually carried out in the field under isolation. Poly- and single crossing by hydroponic culture of panicles in the green house is useful for small amount of seed production such as experimental strain and half-sib families. Equal numbers of panicles with one or two leaves are collected from each selected plant in the field just before flowering ,and cultured in water without any nutrients for about 1 month (Figure 1). A current of air by an electric fan is necessary for the pollination in the green house.

Seed yield varies among seed production locations and cultivars. Seed yield of early maturing cultivars is usually higher than those of late maturing cultivars. The location of commercial seed production for cocksfoot cultivars is often different from the location of breeders for those cultivars. Genotype × location interactions between Indiana and Oregon in the USA were significant for seed yield and its

Fig. 1 Polycrossing technique by hydroponic culture of detached panicles in the greenhouse (Photo Y. Sanada)

related traits in 24 polycross progenies (Stratton and Ohm 1989). Two cycles of phenotypic recurrent selection for seed yield based on seed weight per panicle were conducted at four eastern USA locations and one Oregon location using four breeding populations (Barker et al. 1997). Total seed yield increased 111 and 163 kg ha^{-1} $cycle^{-1}$ in two out of four populations in Oregon by convergent–divergent selection (Barker et al. 1997). Seed weight per panicle of five cultivars at three locations in Japan and the USA was positively correlated (Yahagi et al. 2002). These results suggest that seed weight per panicle could be used as selection index for increasing seed yield at both breeders and seed production locations.

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Timothy

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1 Introduction

Timothy (*Phleum pratense* L.), sometimes also called Herd's grass and/or cat's tail, is known as a cool-season perennial forage grass in North and South Americas, Europe, Australia, New Zealand and east Asia. In the EU, timothy ranks third in the certified seed area of forage grasses, after perennial (*Lolium perenne* L.) and Italian ryegrasses (*Lolium multiflorum* L.). It is considered the most important grass in parts of Canada, USA, the Nordic countries of Europe and Hokkaido, the northernmost Japanese prefecture (Berg et al., 1996; Helgadottir and Sveinsson, 2006; McElory and Kunelius, 1995). The amount of timothy seeds sold in eastern Canada is twice as much as that of all other grasses (McElory and Kunelius, 1995), and timothy occupies more than 70% of total grassland in Hokkaido (Tamaki, 2005).

Timothy is a typical bunchgrass, whose height reaches 1 m at the heading stage (Peeters, 2004). It is often used in mixtures with meadow fescue (*Festuca pratensis* Huds.) and/or red clover (*Trifolium pratense* L.) and mostly mown for silage making. Under nitrogen fertilizer input lower than 300 kg N/ha/year, yield level of timothy is higher than that of perennial ryegrass or meadow fescue (Baert et al., 2003).

As compared to other major bunch-type perennial cool-season forage grasses, timothy is very winter-hardy. Its LT_{50} value reaches as low as $-26°C$ in some materials (Höglind et al., 2008). Sustainability (Berg et al., 1996) and preference by cattle and/or horses are further advantages. On the other hand, timothy is less popular in drier regions and less widely used in frequently defoliated conditions because it is less resistant to drought and slower in regrowth (Spedding and Diekmahns, 1972).

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Timothy is cross-pollinating due to self-incompatibility, unless in extreme cases (McElory and Kunelius, 1995; Shimokoji, 1998), and is bred using similar methods as most major cool-season forage grasses. Timothy has great advantages for conducting research on breeding science, because a spaced individual can produce a large amount of seeds (30–50 g or about 10^5seeds) in the following year of transplantation (Furuya et al., 1996; McElory and Kunelius, 1995; Tamaki, 2005), and because it is easy to establish test fields, to transplant and/or propagate elite clones and to keep them for decades.

2 Origin and Systematics

2.1 Systematics

Though the taxonomy of *Phleum* has not been completely fixed (Kula et al., 2006), it is widely accepted that two important, closely related and partially cross-fertile groups exist in this genus. The first group, having wild species *Phleum alpinum* L. and *Phleum commutatum* Gaudin, contains diploid ($2n = 2x = 14$) and tetraploid ($2n = 4x = 28$) forms. The tetraploid spreads in Europe, Asia, North and South Americas but the diploid exists only in European mountains. The second group, having more adaptability to lower elevation, contains hexaploid ($2n = 6x = 42$) *P. pratense* L. as well as diploid, tetraploid and octoploid forms (Stewart et al., 2008).

2.2 Genome Construction and Biological Origin

A controversy has not been settled yet on the genome construction of hexaploid *P. pratense*. Though some of early cytogeneticists thought it was allohexaploid (Nordenskiold, 1945), the autohexaploid hypothesis has generally been accepted for decades (Wilton and Klebesabel, 1973; Cai et al., 2003) because little difference has been found among its three genome sets. Resent research, however, supports the hypothesis of autoallohexaploid. Cai and Bullen (1991, 1994) suggested that the three genome sets can be classified into two types, one of which is close to *P. alpinum*. Stewart et al. (2008) examined inter- and intraspecific differentiation in cytoplasmic and nuclear DNA's of the genus and found that the hexaploid *P. pratense* today widely cultivated is partly close to a diploid belonging to *P. alpinum* group and hypothesized that it originated from the Balkans glacial refuge.

2.3 Origin of Cultivation

The origin of timothy cultivation is thought to be distant from its biological one. According to Berg et al. (1996), the first records on intentional timothy cultivation is in the early 18th century in North America, to which it is thought to have

been introduced by European colonists in 1700s, perhaps unintentionally. There are records that John Herds recommended it as a forage grass after he found it in New Hampshire about 1710; and Timothy Hansen brought the seeds intentionally from England to USA about 1720. Later, the cultivated strains were re-introduced from USA to Europe. However, by that time, timothy had already been cultivated also in Sweden under the name 'Angkampe' (McElory and Kunelius, 1995).

3 Variety Groups

3.1 Ploidy

Cultivated forms of *P. pratense* are predominantly hexaploid, though some diploid cultivars have also been bred. These are referred to as *Phleum nodosum* in the list of OECD (2008) and as *P. pratense* subsp. *bertolonii* by Stewart et al. (2008). On the OECD list of 2008, a total number of 173varieties of *P. pratense* are listed.

3.2 Maturity

Timothy varies remarkably in maturity. In Hokkaido lying between 41 and 46°N latitudes, its maturity is classified into four groups; extremely early, early, medium and late. The heading dates of extremely early-maturing 'Kunpu' and late-maturing 'Hokusyu' are June 9 and July 4, respectively (Shimokoji, 1991). Furthermore, it is known that some introduced materials classified as 'extremely late' cannot reach heading at all because of insufficient day length. Though the variation of maturity is continuous, different base populations have been established for each of the maturity groups. Early-maturing material mostly originates from old local varieties, while introduced materials from Europe are important in the late-maturing group. In north-western European countries lying between 45 and 55°N latitudes, the latest varieties start heading about 3 weeks later than the earliest ones. The late varieties are mainly pasture types.

3.3 Classification in Nordic Countries

Five zones are defined in Nordic countries for testing performance of timothy varieties depending on the latitude (Bjornsson, 1993). The day length requirements are very different among latitudinal ecotypes and affect growth and frost tolerance. The plant height of variety 'Grindstad' (latitudinally adapted to 59.5°N) responds within a narrow photoperiodic interval from 9 to 15 h with a maximum sensitivity of 12.5 h, while that of variety 'Engmo' (latitudinally adapted to 69°N) responds nearly linear over the photoperiodic range from 9 to 24 h with a maximum sensitivity of 15 h (Wu et al., 2008). The two varieties differed greatly in LT_{50} for frost

tolerance, with −15.5°C for 'Grindstad' and −26°C for 'Engmo' (Höglind et al., 2008). 'Grindstad' headed only 1 day earlier than 'Engmo' under a 24-h photoperiod (Larsen and Honne, 2001).

4 Genetic Resources and Utilization

4.1 Local Varieties

Locally cultivated timothy populations were the initial basis of the breeding material, because its wide cultivation in Europe and North America since 19th century led to the establishment of many local varieties or landraces (McElory and Kunelius, 1995). Alderson and Sharp (1995) report that American varieties 'Clair' and 'Vantage', Canadian 'Champ' and 'Richmond', Dutch 'Barmoti' and 'Barvanti', Icelandic 'Korpa', Italian 'Toro' and Swedish 'Alexander' and 'Bottnia II' were bred from local materials from the respective countries. In Japan 'Senpoku' and 'Hokuren' had been bred from local materials from Hokkaido (Maki, 1985; Shimokoji, 1991).

In Norway, the most sold variety is still 'Grindstad', a landrace owned and maintained by a farmer. The seeds harvested from his meadow after 2 years of forage harvest are used to establish new seed fields serving to produce basic seed for multiplication in nearby regions (Marum, 1999). Local populations are still of interest in breeding varieties for northern climatic areas where new cultivars must survive a long period with negative carbon balance and winter stresses, combined with intensive growth during a short period with mostly 24 h photoperiod (Larsen and Honne, 2001).

4.2 Introduced Materials

Materials introduced from Ukraine became the parents of the Japanese variety 'Hokuo' (Alderson and Sharp, 1995). Japanese 'Hokusyu' was bred from materials having European and New Zealand origins (Ueda et al., 1977a). However, more frequently, introduced materials were combined with local ones. Varieties having been bred in this way are Japanese 'Nosappu', 'Kunpu', 'Kiritappu' and 'Natusakari', Swedish 'Argus' and Canadian 'Bounty' (Alderson and Sharp, 1995; Furuya et al., 1992a, Masutani et al., 1981; Ueda et al., 1977b; Yoshizawa et al., 2005).

4.3 Progeny of Elite Materials

Commercial varieties or experimental populations having been developed in previous breeding programmes are popular resources for new programmes in organizations where timothy breeding has been conducted for decades. Canadian 'Salvo'

(released in 1980) and ‘Mariposa’ (1984) were selected from their predecessors, ‘Champ’ (1967) and ‘Richmond’ (1976), respectively (Alderson and Sharp, 1995). All of the seven parental clones of Japanese synthetic variety ‘Akkeshi’ (1992) came from ‘Senpoku’ (1969) or from experimental strains having been developed in the same organization (Furuya et al., 1992b). Also in current breeding programmes in Norway, advanced breeding material and varieties well adapted to the climate are the main genetic resources.

4.4 European Genebanks

The Nordic Genebank (http://www.nordgen.org/ngb) has a collection of 674 accessions of *P. pratense* (July 2009) from the Scandinavian countries (Denmark, Finland, Iceland, Norway and Sweden). Part of this collection is well characterized for morphological and agronomic traits (Fjellheim et al., 2007). The European Central ECPGR *Phleum* database is maintained by the Nordic Genetic Resource Centre (NORDGEN). It contains passport data on 5439 (July 2009) accessions of 16 *Phleum* taxa stored in 30 European genebanks. The collection contains accessions from 40 countries or regions, 4 of them outside Europe, and includes commercial varieties (660), local cultivars (232), wild or semi-wild materials (4112), breeding or research materials (18) and those of unknown types (418). Larsen and Marum (2006) found a clear relationship between the climate where an accession was collected and winter hardiness, indicated by percent ground cover in the spring.

5 Major Breeding Achievements

5.1 Disease Resistance

Improved disease resistance is the most obvious achievement of past timothy breeding programmes both in Europe and in Japan. In Europe, leaf disease resistance is considered among the traits where progress was evident (van Waes et al., 2008; Plantum, 2008). In Belgium, two commercial varieties have been widely spread over the world: old ‘Erecta’ (released in 1952) and newer ‘Comer’ (1997); the latter has better leaf spot resistance than the former. In Japan, one of the most successful achievements in the past timothy breeding was improved resistance to the purple spot disease caused by *Cladosporium phlei* (Gregory) de Vries. The region where timothy cultivation spreads most widely is humid (100 mm/month) and chilly (13 – 21°C) during summer. Therefore the damage from the disease is often so severe that it may bring the failure of grassland establishment or disastrous yield decrease (Shimokoji, 1991). The resistance of varieties to this disease has been improved from medium (score 5) in the 1970s to high (score 7) around 2000.

5.2 Yield

In Japan, forage yield improvement has been thought important to raise dairy and livestock productivity, and yield improvement is considered one of the major achievements in the past timothy breeding. Between 1969 and 2004, a steady yield increase of 0.32% per year can be calculated from the data of Shimokoji (1998). This was mainly related to improved re-growing vigour.

On the other hand in Europe, yield improvement seems to have been a hardship. In all the north-western European lists, some varieties more than 30 years old still belong to the recommended varieties. Recent European varieties have made very little progress in annual dry matter yield (DMY), except for early development. Comparing with the old Norwegian variety 'Grindstad', the newest 'Lidar' has a better winter survival but the same productivity. Belgian 'Comer' yields only 3% more DM than its predecessor 'Erecta' released over 40 years earlier. 'Snorri', a new Nordic variety showed good yield stability in all the Nordic regions but did not show clear yield advantages over some control varieties (Helgadottir and Kristjansdottir, 2006).

5.3 Other Characteristics

Japanese breeding programmes were successful in markedly widening the span from early- to late-maturing varieties. This helped farmers to expand forage production by extending the potential harvesting period for conservation (Shimokoji, 1991). In Europe, progress has also been recognized in traits such as digestibility, winter hardiness, persistency and sward density (BSA, 2007; Suter et al., 2008).

6 Specific Goals in Current Breeding

6.1 Yield and Disease Resistance

These two traits remain important in most current breeding programmes. In Europe, they are among the top three criteria in national official trials determining the value for cultivation and use (VCU). Yield includes both total annual DMY and good yield in spring and late summer. Disease includes leaf spot diseases like Helminthosporium (*Drechslera*) and Heterosporium (*Cladosporium*) in north-western and central Europe, as well as snow moulds (mainly *Typhula ishikariensis*, sometimes *Sclerotinia borealis*) in Scandinavia.

6.2 Persistency

It is also highly important in official European VCU trials and often related to winter hardiness (frost tolerance) and to tolerance to frequent defoliation by cutting or

grazing. Northern varieties have higher water-soluble carbohydrate (WSC) concentration during autumn than more southern varieties (Larsen and Marum, 2006), and this may contribute to their better winter hardiness.

6.3 Nutritive Values

Though timothy is regarded of high forage quality (McElory and Kunelius, 1995), traits such as digestibility and protein content still attract much attention from breeders for even higher dairy and livestock productivity. Dry matter digestibility (DMD) decreases rapidly (2 – 7 g/kg DM/day) with increasing maturity. The indigestible part of the neutral detergent fibre (NDF) is considered an important parameter in the new Nordic system for evaluating fodder quality. Marum et al. (1994) showed that it is possible to obtain significant responses to selection for digestibility and protein content, but it has to be paid for by a reduction in DMY. However, recent studies of Bélanger et al. (2006) confirm that improvement in the digestibility of the structural component has the potential of improving forage DMD with no negative impact on DMY. Water-soluble carbohydrate (WSC) concentration is also an index interesting for quality-oriented breeders. A large variation was found in this index among timothy varieties (Jonaviciene et al., 2008). Ashikaga et al. (2008) indicated that indices for digestibility and WSC concentration have high narrow-sense heritability. Near-infrared reflectance spectroscopy (NIRS) is widely used to analyse forage quality.

6.4 Lodging Resistance in the First Crop

Lodging resistance has been considered a factor for stable, high yield in many crops (Tamaki, 2005). In timothy cultivation, lodging increases the proportion of dead or dying plant parts and brings about negative effects on quality (Gustavsson, 2006), especially in the first crop. In Hokkaido, where lodging resistance has been one of the most important breeding targets, ‘Aurora’ and ‘Natusakari’ are examples of commercial varieties having improved lodging resistance (Figure 1) (Shimokoji, 1994; Yoshizawa et al., 2005). Tamaki et al. (2002a) found that lodging resistance in the different growth stages of the first crop should be regarded as different traits from each other, and that its narrow-sense heritability in each stage is high.

6.5 Competitiveness and Others

Competitiveness after the first cut has been among the major breeding targets in Hokkaido because timothy is often outcompeted by accompanying forage legumes or by weeds after the first cut. Tamaki et al. (2002b) indicated that narrow-sense heritability of this trait is high, but that it can only be precisely evaluated under conditions of actual competition with legumes or weeds. Individual selection from

Fig. 1 Lodging of 'Natusakari' (*left*, released in 2004) and of 'Hokusyu' (*right*, 1980) at the first cut

Fig. 2 Spaced timothy plants in the second flush under conditions of competition with white clover (Photo H. Tamaki)

breeding nurseries with competing white clover (Figure 2) has been found effective to improve competing ability. Nitrogen use efficiency for organic and low input production, ability for quick establishment, sward density and drought resistance are other traits of interest.

7 Breeding Methods and Specific Techniques

7.1 Breeding Methods

7.1.1 Mass Selection and Synthetic Varieties

In recent years, open pollinated varieties obtained by mass selection have been widely replaced by synthetic varieties. Synthetic varieties are renowned for their high yield levels. Among them are Japanese 'Nosappu', 'Kiritappu' and 'Horizon',

though the most outstanding is Canadian 'Climax' (Ueda et al., 1977b; Furuya et al., 1992a; Alderson and Sharp, 1995, Tamaki et al., 2002c). Due to the longevity of the species, mother clones can easily be kept alive during progeny testing. As an alternative, surviving plants taken from the progeny test can be used to compose the synthetics (Reheul et al., 2003). A further modification of this method is described as maternal line selection.

7.1.2 Maternal Line Selection Combined with a Progeny Test

Though there are several versions of this method, the one described here is frequently adopted in Hokkaido since 1990s. The objectives are the same as in the scheme 'among- and within-family selection' presented by Casler and Brummer (2008). As shown in Figure 3, it allows breeders to conduct individual selection and a polycrossed progeny test simultaneously. When elites are selected as the parents of the new variety candidates, results from the parallel progeny tests of their mother clones will be referred to evaluate yield levels or other traits of low narrow-sense heritability. As individual selection and progeny tests can be conducted simultaneously, the length of one breeding cycle can be as short as that of mass selection. Though it is painstaking to conduct the two large-scale field tests simultaneously and continuously, breeders can operate two cycles in this method while only one can be done in variety synthesis. A number of Japanese variety candidates developed using this method will be registered in early 2010s.

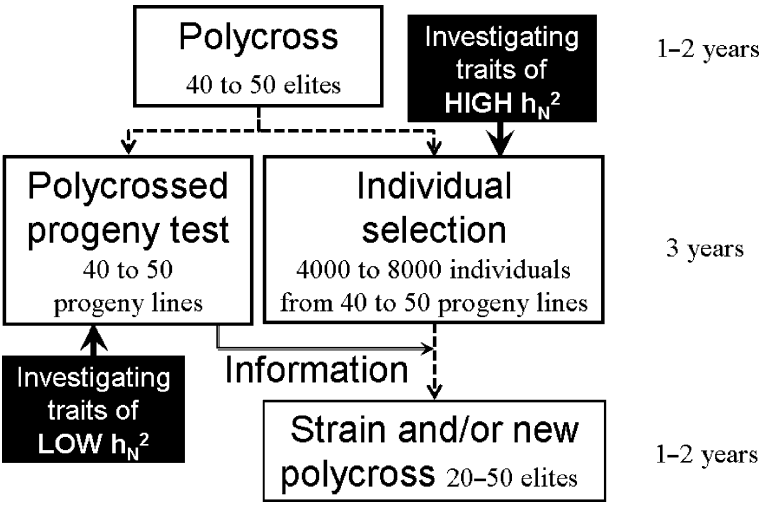

Fig. 3 Scheme of maternal line selection combined with a progeny test

7.1.3 Clone and Strain Synthesis (CSS)

CSS was proposed by Tamaki et al. (2004b) for improving yield rapidly by exploiting not only GCA but also specific combining ability (SCA). As shown in Figure 4, variety candidates are synthesized from two seed parental clones (A and B in Figure 4) topcrossed to a pollen parental strain (or population) (P). Yielding ability in syn-2 partly depends on SCA between A and B, A and P and B and P. Tamaki et al. (2009) confirmed that some timothy synthetics developed in CSS have significantly higher yield levels in syn-2 than a commercial variety to which the parental clones had been topcrossed.

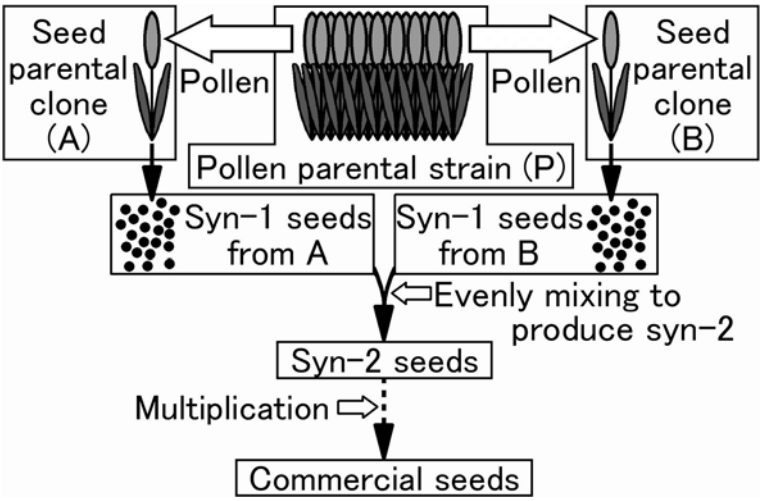

Fig. 4 A typical scheme of clone and strain synthesis (CSS)

7.2 Specific Techniques

7.2.1 Purple Spot Disease Inoculation Test

This test was developed by Tsutsui et al. (1990) to effectively improve resistance. The test involves the following steps: (1) 6.5-leaf-age seedlings are incubated in the dark at 15°C for 72 h, (2) liquid containing 4–6 $\times$ 10^6 spores is sprayed into the seedlings, (3) the seedlings are incubated for another 72 h in the dark at 15°C with 100% humidity, (4) the seedlings are set in natural day length at the same temperature for several days and (5) their resistance to the disease is judged by evaluating the extent of damage.

8 Integration of New Biotechnologies in Breeding Programmes

Little research seems to have borne fruit in integrating new biotechnologies with timothy breeding. The polyploid nature of timothy probably made it less attractive as a material of marker-assisted breeding. Ogawa et al. (2002) searched for

RFLP and AFLP markers closely linked to purple spot disease resistance, and found one in a pseudo-testcross F1 population. However, it did not effectively explain the resistance in other populations (Cai, personal communications).

In Finland, DNA marker tools are developed for better digestibility and disease resistance (Manninen et al., 2006). Molecular techniques for characterization of populations and clones are investigated to identify genetically distant genotypes as the basis for improved heterosis. Selection of these genotypes may form the basis of new synthetic varieties (Fjellheim et al., 2007; Larsen and Marum, 2006).

8.1 Relatedness of Varieties Based on Molecular Markers

Guo et al. (2003) examined differentiation of 38 varieties from 15 countries by RAPD and universally primed-PCR. Relatedness among varieties reflected geographical proximity or countries in which they had been bred. Pulli et al. (2003) discuss that the results also accord with those from artificial frost resistance tests. However, they failed to detect major differences in pedigree of varieties bred in Hokkaido. For example, extremely early-maturing 'Kunpu' bred from a local variety from Hokkaido and an American variety 'Clair' (Masutani et al., 1981) and late-maturing 'Hokusyu', having European and New Zealand origins (Ueda et al., 1977a), were classified as very close by Guo et al. (2003).

9 Seed Production

Timothy requires a vegetative development and a long-day induction for flowering. Vernalization requirement is not obligatory but quantitative. Seeds can be produced in most areas where timothy is grown as forage (McElory and Kunelius, 1995) and its seed productivity is relatively high (Tamaki, 2005). These advantages have contributed to the establishment of many local varieties before contemporary large-scale breeding programmes were started. Current commercial seed production for successful varieties, however, is concentrated in some specific regions, often far away from the region of origin and utilization as forage. In North America, most certified seeds are harvested in the higher rainfall areas in the Northern Great Plains and the Peace River region of Alberta (McElory and Kunelius, 1995). Almost all commercial seeds of Japanese varieties are produced not in Japan but in Oregon, USA (Iwabuchi, personal communications). Different from other forage grass species, seed multiplication outside the area of development does not appear to alter population structure or forage yield in timothy (Simon and Kastenbauer, 1979).

Commercial seeds of European varieties are produced in regions less distant from those of their cultivation. In the EU, 5780 t (average over 2004–2006) of commercial seeds are produced from 16700 ha (average over 1998–2007). Finland, Sweden and Germany occupy 40, 23 and 19% of the production, respectively. In 2007, 19422 ha

of *P. pratense* and 80 ha of *P. nodosum* or *bertolonii* were accepted for seed certification in the EU (http://ec.europa.eu/agriculture/agrista/2007). Norway, non-EU member, produces another 1000 t per year on average.

Seed productivity is also a trait of interest for breeders because it can be a key factor of the varieties' commercial success. The ripening period of earlier maturing materials tends to be longer than later ones, which leads to larger seed size and higher seed yield of the former (Furuya et al., 1996). Seed productivity within the same maturity does not depend on component traits such as seed size, number of ears per plant or of caryopses per ear (Tamaki et al., 1998), but probably on ripening rate (germinable caryopses)/(total caryopses) (Tamaki, 2005). Tamaki et al. (2004a) found that seed productivity was highly heritable.

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Bluegrasses

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1 Introduction

Bluegrasses, also known as meadowgrasses, represent one of the most economically important and agronomically useful groups of grass species, excluding those for human consumption (Soreng and Barrie 1999). In temperate climates of the world, bluegrasses are utilized as fundamental components of pastures, meadows, and cultivated turfs, including lawns, sports fields, and golf courses. Bluegrasses are also valuable crops for both seed and sod production and they serve an important function as native and naturalized plant species for soil stabilization and enhancing ecological diversity in land restoration.

1.1 Agronomically Relevant *Poa* Species

Equal to their diverse agricultural and ecological utility, the bluegrasses also exhibit a wide range of biological diversity, particularly in regard to reproductive biology. Most species possess hermaphroditic flowers but gynomonoecious, gynodioecious, and dioecious species are present as well (Anton and Conner 1995). Modes of reproduction include self-pollination, cross-pollination, and facultative apomixis. Because reproductive biology essentially dictates our breeding methodologies, breeders need to be fully aware and understand the mode of reproduction of the bluegrass species they are breeding. In addition, many of the bluegrass species have retained an ability to recognize foreign pollen from other bluegrass species and so breeders should also be aware of the extensive possibilities for interspecific hybridization. In nature, interspecific hybridizations have resulted in a convoluted reticulation of genome mixtures within the *Poa* genus and a continuum of morphological characteristics among species. The genomic mixing and taxonomic reticulation are so extensive within the bluegrasses that G.L. Stebbins, the eminent plant evolutionary geneticist, whose early professional ambition was

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to become a grass breeder, was fond of remarking that he "wouldn't be surprised if someday, someone discovered that all bluegrass species were actually just members of a single huge polyploid complex" (Stebbins 1950; Stebbins pers. commun. ca. 1987).

Kentucky bluegrass (*Poa pratensis* L.) is the botanical-type species for the genus *Poa* and is commonly recognized as the most economically important and widely utilized bluegrass species for both forage and turf (Wedin and Huff 1996). Kentucky bluegrass represents over 90% of all available cultivars and germplasm accessions of agronomically important bluegrass species maintained in repositories worldwide (Table 1). Annual bluegrass (*Poa annua* L.) may be the most widely distributed of the bluegrass species and also has a large economic impact in the turf industry both as a weed to be controlled and as a valuable playing surface on golf course putting greens (Huff 2003). Other agronomically and ecologically important bluegrasses throughout the world include Canada bluegrass (*P. compressa* L.), wood bluegrass (*P. nemoralis* L.), fowl bluegrass (*P. palustris* L.), rough bluegrass (*P. trivialis* L.), supina bluegrass (*P. supina* Schrad.), alpine bluegrass (*P. alpina* L.),

Table 1 Various attributes of agronomically important bluegrass species

Common name	Latin name	Chromosomes (x=7)	Reproduction[1]	Phylogenic clade[2]	Accessions[3]	Cultivars[4]
Kentucky	*P. pratensis*	18–156	fac. aposp. apomixis	PoM	6,689	261
Canada	*P. compressa*	35–56	fac. aposp. apomixis	SPOSTA	58	4
Wood	*P. nemoralis*	42,56,70	fac. diplo. apomixis	SPOSTA	67	6
Fowl	*P. palustris*	14,28,42,63	obl. diplo. apomixis	SPOSTA	59	1
Sandberg	*P. secunda*	62–100	fac. aposp. apomixis	SPOSTA	19	1
Rough	*P. trivialis*	14	obl. sexual cross-pollinated	SPOSTA	68	14
Texas	*P. arachnifera*	(42),56	obl. sexual dioecious	HAMBADD	22	1
Annual	*P. annua*	28	obl. sexual self-pollinated	BAPO	41	1
Supina	*P. supina*	14	obl. sexual cross-pollinated	BAPO	9	1
Alpine	*P. alpina*	22–46	diplo.apomictic seed and pseudovivipary	BAPO	43	1
Bulbous	*P. bulbosa*	28,35,42	seed (apomictic?) and pseudovivipary	BAPO	173	1

[1]Fac. = facultative; obl. = obligate; aposp. = aposporous; diplo. = diplosporous.
[2]Follows Gillespie and Soreng, 2005.
[3]Source: Bioversity http://www.bioversityinternational.org/
[4]Source: OECD List of varieties eligible for certification 2007/2008 http://www.oecd.org/document/14/0,3343,en_2649_33905_2485070_1_1_1_1,00.html; Author experience.

and bulbous bluegrass (*P. bulbosa* L.). Two additional species with regional importance in North America include Sandberg bluegrass (*P. secunda* J. Presl.) and Texas bluegrass (*P. arachnifera* Torr.). Due to its overwhelming importance, this chapter will focus primarily on the breeding of Kentucky bluegrass. Detailed reviews for the breeding and genetics of other bluegrass species include Texas Bluegrass (Read and Anderson 2003), rough bluegrass (Hurley 2003), supina bluegrass (Burghrara 2003), and annual bluegrass (Huff 2003).

1.2 Apomixis in Bluegrasses

The central over-riding theme which governs the overall biology and every aspect of breeding Kentucky bluegrass is its facultative apomictic mode of reproduction. Advanced knowledge of Kentucky bluegrass reproduction is a prerequisite for breeders but requires time and patience to attain. Even the terminology associated with apomixis has been described as a "quagmire" by Asker and Jerling (1992) and the developmental complexities of apomixis make the categorical placement of *Poa* species into particular forms of apomixis difficult (see Savidan 2000). To make matters worse, our understanding of the genetic control and evolutionary origins of apomixis is limited. Apomixis is a genetically complicated trait and physically elusive by occurring within the ovule and as such, the more it is investigated, the more complicated it becomes. Nevertheless, the phenotypic expression of apomictic reproduction in Kentucky bluegrass appears to be simply inherited and environmentally stable (Mazzucato et al. 1996) and thus breeders have been successful in manipulating and utilizing apomixis in the development of commercial cultivars of Kentucky bluegrass.

Apomixis is a developmentally complex form of asexual reproduction resulting in progeny plants that are genetically identical to the seed-bearing parent and is commonly referred to as "seed without sex." In the absence of sexual recombination, apomictic plants are capable of harboring and propagating odd ploidy levels and aneuploid chromosomal abnormalities that would render sexually reproducing plants completely sterile due to problems associated with the reductional division process of meiosis. For example, chromosome numbers in Kentucky bluegrass exhibit a nearly continuous distribution of aneuploidy ranging between 24 and 124 chromosomes with skewed distributional modes in the 49–56, 63–70, and 84–91 chromosome number classes (Nielsen 1945, 1946, Love and Love 1975). In addition, chromosome numbers outside of this range have been observed (Muntzing 1940, Kiellander 1942, Huff and Bara 1993). Apomixis also enables plants to propagate intact, odd ploidy levels resulting from interspecific hybridization. In the presence of apomixis, assimilating genomes through interspecific hybridization events results in many genomically different and genetically distinct phenotypes within an apomictic species. For example, some 45 "species" have been described within the apomictic complex known as Sandberg bluegrass (Kellogg 1990). Given the lack of regular recombination and segregation in apomictic plant species, genetic

gains from selection will primarily be based on epistatic gene interactions rather than from accruing additive genetic effects as is typical in sexually reproducing plant species. Thus, attaining crop improvement within apomictic species is performed without the aid of predictive powers associated with Mendelian inheritance and quantitative genetics. As such, breeders of apomictic species need to adapt their breeding methodology which requires detailed knowledge of the involved form of apomixis. Recent reviews of apomixis include those by Savidan (2000), Bicknell and Koltunow (2004), Ozias-Akins (2006), Ozias-Akins and van Dijk (2007), and Naumova (2008).

Kentucky bluegrass has a form of apomixis known as facultative pseudogamous apospory in which a nucellar cell mitotically develops into an unreduced embryo sac that continues development autonomously through parthenogenesis but requires pollen fertilization of the central cells for proper endosperm development (Muntzing 1933, Tinney 1940, Grazi et al. 1961). In an F1-segregating population of size n=38 between a sexual and an apomictic Kentucky bluegrass, Albertini et al. (2001) were able to uncouple the two main components of apomixis: apospory and parthenogenesis. Parthenogenesis ranged from 0.0 to 92.9% within the segregating population and apospory was observed in two non-parthenogenic individuals. The authors concluded that a minimum of four genes were involved in the expression of parthenogenesis in the sexual parent and one in the apomictic parent for this particular segregating population. Thus, the facultative nature of apomixis in Kentucky bluegrass implies that various components of the apomictic process occasionally break down resulting in progeny plants that are distinctly different from the seed-bearing parent. These distinctly different progenies are referred to as "off-types" or "aberrants." The overall frequency of aberrant progeny results from the variable frequencies of reduced (meiotic) and unreduced (aposporous) egg formation and whether or not these eggs are fertilized (Table 2). As such, different aberrant progeny may have distinctly different genetic origins (Huff and Bara 1993).

Several other agronomically important bluegrass species also reproduce by means of an apomictic system and thus the principles of breeding these species will be similar to that of Kentucky bluegrass though the details of the involved apomictic system may be different and so may require specific alternations. Canada bluegrass and Sandberg bluegrass have an apospory apomictic system similar to

Table 2 Genetic origins of progeny from apomictic bluegrasses. Female gametes develop through either apomeiosis (apospory and/or diplospory) or meiosis (reductional division). Male gametes either contribute (via self- or cross-fertilization) or not (pseudogamy) to the genetic makeup of the progeny. Theoretical ploidy levels are in parentheses with the value of n ranging approximately from 18 to 40 chromosomes

Male/Female	Pseudogamy (–)	Self (n)	Cross (n)
Apomeiosis ($2n$)	Apomict ($2n$)	BIII ($3n$)	BIII ($3n$)
Meiosis (n)	Polyhaploid (n)	BII ($2n$)	BII ($2n$)

Kentucky bluegrass (Kellogg 1987). Wood bluegrass and fowl bluegrass possess a diplosporous form of apomixis (Naumova et al. 1999) where archegonial cell initials mitotically divide to form unreduced embryo sacs.

Some species of bluegrasses are capable of producing either seed or vegetative bulblets within the florets (a type of apomictic vivipary referred to as pseudovivipary). Two species that regularly produce both bulblets and seed are bulbous bluegrass, which reproduces mostly by seed within its native range (ex. cv. 'Nevskii', *P. bulbosa* L. ssp. *nevskii* (Roshev. ex Ovcz.) Tzvelev) but in North America reproduces primarily by bulblets formed within the florets, (Novak and Welfley 1997) and alpine bluegrass, which regularly reproduces by either seed or through bulblets (*P. alpina* var. *vivipara* L.) throughout its range.

Agronomically important bluegrasses having solely sexual reproduction include rough bluegrass and supina bluegrass which are hermaphroditic outcrossers, Texas bluegrass which is a dioecious outcrosser, and annual bluegrass which is predominantly self-pollinated.

2 Origin and Systematics

The bluegrass genus *Poa* is the largest genus of the grass family *Poeae* with 500+ species and has a monophyletic origin (Gillespie and Soreng 2005). Bluegrasses have a Eurasian center of origin but are adapted to a wide range of climates including temperate, boreal, and polar regions of the world (Soreng 1990). According to Grun (1954), the *Poa* genus contains a few diploid ($2n=2x=14$) species, some tetraploid species but most species result from highly complex polyploidy formations. Bluegrass species are taxonomically challenging because they collectively exhibit an ability to form interspecific hybridizations resulting in overlapping morphological characteristics and worldwide distribution due to the buffering capacity of polyploidy. In addition, new species of bluegrass are known to endemically develop in many parts of the world (see refs. in Gillespie and Soreng 2005). Even taxa above the species level are thought to have a hybrid origin (Soreng 1990). Thus, in addition to the bluegrasses being important agronomic grasses, they also play an important role in enhancing our understanding of polyploid species formation and evolution.

Individual bluegrass species often lack discreet morphological characteristics required for their taxonomic identification (Clausen 1961). In agricultural practice, there are particular morphological features which are useful in the identification of bluegrasses from some of the other co-existing grasses. These characteristics include a pair of prominent, parallel grooves formed by bulliform cells adjacent to the midrib on the adaxial leaf surface. This feature is sometimes referred to as a "train-track" midrib owing to the resemblance of a set of train tracks. In Europe, this feature is also known as a "ski-track" midrib owing to its resemblance to the set of tracks made by cross-country skiers. Bluegrasses also typically possess a leaf tip that resembles the bow of a row boat and are thus referred to as having a "boat-shaped"

tip. A physical test for a boat-shaped tip may be performed by placing a leaf blade between one's thumb and index finger and sliding the thumb over the leaf tip. This action compresses boat-shaped leaf tips to form a v-shaped notch, otherwise the tips remain pointed and are not considered boat-shaped. Bluegrasses have panicle-shaped inflorescences, a membraneous ligule, and lack auricles. Individual species of bluegrasses of agronomic importance are further identified based on growth habit and/or specific features such as the translucent bulliform cells of Kentucky bluegrass or the wrinkled leaf blades of annual bluegrass.

Bluegrasses are also the only grass species to possess cottony hairs on the lemma's callus at the base of their florets, which is dramatically exhibited in Texas bluegrass, although not all bluegrass species exhibit this cottony webbing. Gillespie and Soreng (2005) suggest that this cottony webbing is an effective seed dispersal mechanism and may contribute to the worldwide distribution of *Poa* species compared to other grass genera. However, within the bluegrasses, this correlation seems to break down. For example, annual bluegrass essentially lacks this cottony webbing and has cosmopolitan distribution, Kentucky bluegrass has moderate levels of cottony webbing and has circumpolar distribution, while Texas bluegrass has copious amounts of cottony webbing and distribution restricted to North America. Therefore, it would appear that while cottony webbing may aid in a species distributional advance, the genetics controlling adaptation within the species must also play a critical role for enabling a wide distributional pattern.

The origin of Kentucky bluegrass is most likely Eurasia but it has a circumpolar distribution. The asexual apomictic reproduction of Kentucky bluegrass along with the resulting buffering capacity of polyploidy are likely the important features of its circumpolar distribution (Grazi et al. 1961, Clausen 1961, Kellogg 1990). As mentioned above, the genome of Kentucky bluegrass is a highly complex distribution of polyploidy and aneuploidy which is primarily due to the retention of pollen recognition systems for many of the other bluegrass species and the ability to propagate chromosomal abnormalities through an asexual apomictic reproductive system. Chromosome numbers for any particular individual are typically reported for Kentucky bluegrass using the prefix circa (ca.) rather than an exact number. This is because even among cells of an individual genotype, there may be slight variations in the number of chromosomes transmitted to somatic or germinal daughter cells (Grun 1955). Multivalent formations at metaphase as well as lagging chromosomes at anaphase are commonly observed (Nielsen 1946, Wu and Jampates 1986, Huff and Bara 1993). Interestingly, according to Grun (1955), interspecific hybridization between Kentucky bluegrass and other *Poa* species does not necessarily increase the frequency of multivalent aneuploid chromosome formations. This observation suggests that there is homology among genomes of other *Poa* species and those within Kentucky bluegrass.

Due to the overlapping morphological variation among bluegrass species, molecular marker analysis has proven vital to firmly establish the phylogenetic relationships among bluegrass species. Two recent studies have examined the phylogeny

of *Poa* species and each provides molecular evidence for the reticulation of *Poa* genomes among species.

A study by Gillespie and Soreng (2005) examined 77 *Poa* species plus an additional 10 infraspecific taxonomic units using restriction enzyme digests of five single copy regions of the chloroplast genome. They were able to cladistically analyze 81 maternally inherited haplotypes. Several species were observed to contain more than one haplotype but two species in particular, namely *P. hartzii* and *P. bulbosa*, exhibited distinctly different haplotypes which likely have origins from different phylogenetic clades. Gillespie and Soreng (2005) concluded that, despite the phylogenetic reticulation caused by interspecific hybridization and polyploidy, their molecular phylogenetic analysis closely resembled morphological cladistic relationships and that the *Poa* genus was capable of being organized into five major phylogenetic clades thereby consolidating some of the *Poa* sections: ArcSyl (*Arctopoa+Sylvestres*); BAPO (*Bolbophorum+Alpinae*) (*Parodiochloa+Ochlopoa*); SPOSTA (*Secundae* (*Pandemos* (*Orienos+Stenopoa+Tichopoa+Abbreviatae*))); PoM (*Poa+Macropoa*); and HAMBADD (*Homalopoa+Acutifolae+Madropoa+Brizoides+Austrofestuca+Dioicopoa+Dasypoa*) (see Table 1).

A study by Patterson et al. (2005) examined 22 *Poa* species by analyzing the DNA sequence of two cloned single copy nuclear genes: *trx* and CDO504. Even though they examined only one forage type (‘Kenblu’; ca. 50 chromosomes) and one turf type (‘Coventry’; ca. 77 chromosomes) of Kentucky bluegrass, they found that each possessed multiple sequences some of which were more similar to other species of *Poa* than to the other Kentucky bluegrass cultivar. This result illustrates the complex composition of Kentucky bluegrass genomes and that not all Kentucky bluegrasses are created equally. Thus, Patterson et al. (2005) provide a reasonable explanation as to the basis and partitioning of the extensive genetic diversity among cultivars and germplasm accessions encountered by Kentucky bluegrass breeders. Such information will enable breeders to make more informed choices with regard to interspecific hybridizations or to attempt particular hybridizations that they might not have otherwise considered; perhaps even breeding artificial allopolyploid Kentucky bluegrasses by starting at the diploid level. Moreover, the discovery by Patterson et al. (2005) of differences in the ancestral genomic makeup among Kentucky bluegrass cultivars suggests that there may be more than one way to “build” a Kentucky bluegrass.

3 Varietal Groups

For most agronomically important traits one cares to consider, there is generally more variation observed among cultivars of Kentucky bluegrass than among cultivars of nearly any other grass species (Figure 1). This includes morphological characteristics like color, texture, density but many physiological traits as well; for example, nitrogen utilization during fall fertilization is more variable among

Fig. 1 A National turfgrass evaluation trial (NTEP) for Kentucky bluegrass exhibits extensive variation among cultivars. Kentucky bluegrass typically displays more trait variation among cultivars than any other cool-season grass species (Photo D. Huff)

Kentucky bluegrass cultivars than among different cultivars of tall fescue or perennial ryegrass (Liu and Hull 2006). Such extensive variability among cultivars of Kentucky bluegrass is potentially the result of their different genomic components propagated by apomictic reproduction.

In order to categorize the natural variation observed within his breeding program, C. Reed Funk, Rutgers University, began ascribing cultivars and breeding lines of Kentucky bluegrass to specific categories or types. Murphy et al. (1997) and refinements by Bonos et al. (2000, 2002) have successfully provided a more formal structure to Funk's original observational categories of Kentucky bluegrass cultivar diversity. In common practice, the actual number of categories of Kentucky bluegrass varies, depending on the need for specificity among end users, and may contain as few as five broad categories (see http://www.ipm.iastate.edu/ipm/schoolipm/node/28) or as many as 16 distinct categories including "hybrid types" and "grazing types" (Table 3).

In natural grassland systems, successful genotypes of Kentucky bluegrass often dominate an ecosystem aided by the true-to-type apomictic breeding system and lack of genetic recombination and subsequent segregation (van Treuren 2008). The same may be said of commercial cultivar development. Commercially successful genotypes that possess the desirable combination of high quality and seed yield are emulated by other breeders such that numerous types are developed which appear very similar to the successful cultivar. Thus, some Kentucky bluegrass categories are based on a type cultivar or group of cultivars that all appear similar. For example, the Cheri types all resemble the cultivar Cheri while the BVMG types are based on the cultivars Baron, Victa, Merit, and Gnome which all share similar growth and performance characteristics. Other categories are based solely on agronomic performance, such as the Aggressive types or on ecological adaptation such as the Mid-Atlantic ecotypes. Consequently, cultivars within the cultivar-specific categories tend to lack significant genetic variation (Figure 2) while those within agronomic or ecological

Table 3 Varietal groupings of Kentucky bluegrass following Murphy et al. (1997) and Bonos et al. (2000, 2002)

Types	Characteristics exhibited	Examples
Compact type	Low compact growth habit, long winter dormancy, late spring green up, very good resistance to leaf spot and stripe smut, very attractive turf quality	Blackstone, P-105, Ram 1, Nugget, Glade, Mooonlight, Alpine, Wildwood, Blue Max, Indigo
Midnight type	A compact type with dark green color, excellent turf quality, but susceptible to powdery mildew; resembles Midnight	Midnight, Rugby II, Liberator, Award, Nu-Glade, Quantum Leap, Total Eclipse, Impact, Odyssey, Courtyard, Beyond, Perfection, Tsunami, Chicago II, Arcadia, Freedon II, Midnight II, Barrister
America type	A compact type with finer leaf texture and higher shoot density than Midnight types, moderate winter dormancy, with good resistance to dollar spot, leaf spot, stripe smut and most races of powdery mildew; resembles America	America, Unique, Bedazzled, Apollo, Goldstar, Lakeshore, Brilliant, Langara, Showcase
Aggressive type	Aggressive lateral growth, high shoot density, wear tolerance, fast sod formation, other characteristics vary	A-34, Touchdown, Limousine, Northstar, Princeton 104, Avalanche, Julius
CELA type	Early spring green up, moderate to good winter color, moderate to good resistance to leaf spot and stripe smut, less seed head formation under mowed conditions than Bellevue types	Challenger, Eclipse, Liberty, Adelphi, Jefferson
Cheri type	Medium low growth habit, medium to course leaf texture, medium shoot density, moderate winter dormancy, moderate resistance to leaf spot, good resistance to stripe smut, moderately susceptible to dollar spot; resembles Cheri	Cheri, Sydsport, Cobalt, Serene
Julia type	Medium to dark green color, medium leaf texture, good wear tolerance in European tests, early spring green up, good resistance to leaf spot and stripe smut, susceptible to dollar spot and brown patch; resembles Julia	Julia, Ikone, Caliber
BVMG type	Medium low growth, medium goof turf quality, medium leaf texture, and shoot density, stemmy seed head formation under mowed conditions, poor winter color, long winter dormancy, moderate to poor summer heat stress recovery, moderate resistance to leaf spot, susceptible to stripe smut and billbugs, good seed yield	Baron, Victa, Merit, Gnome, Abbey, Fortuna, Crest, Marquis, Kelly, Nassua, Blue Star, Raven, Baronette, Blue Chip, Cannon, Dragon, Goldrush, Envicta, Brooklawn

Table 3 (continued)

Types	Characteristics exhibited	Examples
Shamrock type	similar to BVMG types with moderate winter color, variable summer heat stress tolerance, and good resistance to stripe smut, resembles Shamrock	Shamrock, SR 2100, Champagne, Parkland, Atlantis
Bellevue type	Medium growth habit, medium leaf texture and shoot density, excellent cool temperature vigor, early spring green-up, stemmy seed head formation under mowed conditions, moderate resistance to leaf spot and stripe smut, moderate susceptibility to billbugs, moderate summer heat stress recovery	Trenton, Banff, Classic, Georgetown, Freedom, Haga, Dawn, Suffolk,
Mid-Atlantic ecotype	Medium-high shoot density with vigorous deep extensive rhizome system and superior summer heat tolerance, excellent recovery from stress, variable resistance to leaf spot, moderate winter performance, produce seed early and economically	Eagleton, Livingston, SR 2000, Preakness, Monopoly, Bel 21, Vantage
Wabash type	A Mid-Atlantic subtype with medium dark green leaf color, moderate susceptibility to leaf spot, and above average resistance to bill bugs, resembles Wabash	Wabash
Other Turf types	Not well placed at this time, exhibits characteristics intermediate between two or more of the above types, requires further study to subdivide	Coventry, Bartitia, Allure, Ascot, Rambo, Baronie, Bronco, Seabring, Washington, Estate, Broadway
Common type	Narrow-leaved, erect growth, produce seed early and economically, susceptible to leaf spot, good abiotic stress tolerance, best adapted to high cutting heights; used for permanent pastures, conservation purposes, and low maintenance utility turf	Kenblu, Park, Alene, Huntsville, Ginger, Rhondi, Argyle, Newport, Piedmont, Geary, Garfield
Grazing type	Leafy, erect growth, high productivity	Troy, Lato
Hybrid type (Kentucky × Texas, reciprocal F1's and backcrosses)	In general exhibits improved heat and drought tolerance but may be variable among cultivars (see text for details)	Reveille, Thermal Blue, Solar Green, Thermal Blue Blaze, Dura Blue, Bandera, Spitfire, Longhorn, Fire and Ice, Fahrenheit 90

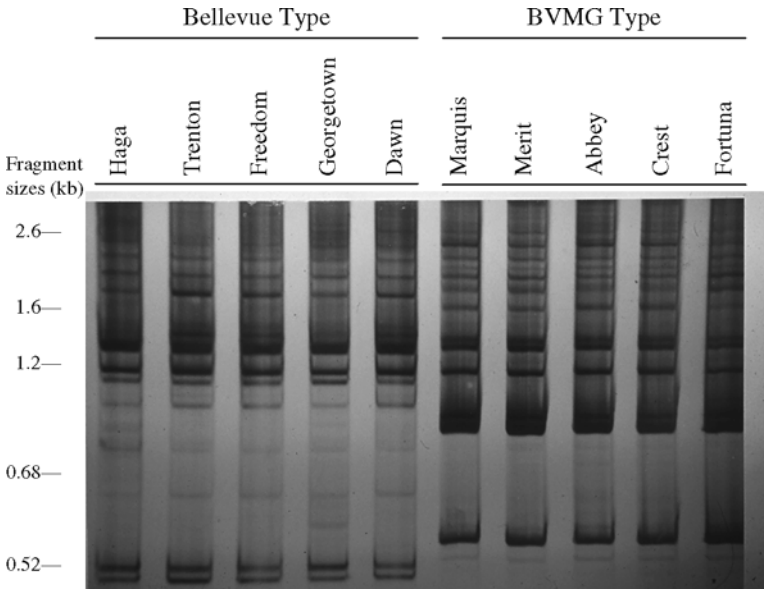

Fig. 2 RAPD marker (OPA-16) profile of five cultivars from each of two different types of Kentucky bluegrass. Cultivars within either Bellevue type or BVMG type appear genetically similar while large differences exist between types

categories tend to be more variable (Huff 2001, Curley and Jung 2004, Eaton et al. 2004).

One important utility of this classification system is to ensure that cultivar blends are properly constructed and do not contain an over-abundance of any one particular type. Due to the genetic uniformity among plants within apomictic cultivars (each cultivar essentially being a single genotype), it is a common agricultural practice to blend different cultivars together in order to promote genetic diversity. In practice, Kentucky bluegrasses are typically established as a blend of three or more cultivars. The purpose of blending cultivars is to increase genetic diversity to combat diseases and enhance stand persistence against extreme environmental stress. However, Brede (2004, 2008) failed to observe the benefits of blending Kentucky bluegrass cultivars. Rather, he observed that such blends frequently exhibit a turf quality that is below that of the best individual component cultivar under various disease pressures and environmental stresses. As a result, Brede (2004, 2008) proposes that establishing mono-stand cultures of individual cultivars might be a viable agronomy practice.

Funk's classification system, along with its continuous modifications, has additional benefits to both the forage and turfgrass industries, particularly by empowering land managers to choose cultivars that are most suitable to their specific management regimes and by enabling breeders to better focus their goals and activities within a breeding program. The large number of categorical types originally

recognized by Funk and his associates is also a testament to the extensive genetic diversity that resides within Kentucky bluegrass.

4 Genetic Resources and Utilization

Genetic resources for Kentucky bluegrass, and many of the other bluegrass species as well, are available at numerous national germplasm repositories worldwide. Most, if not all, of these repositories are linked to the Bioversity International database (http://www.bioversityinternational.org/; Bioversity is the operating system of the International Plant Genetic Resources Institute, IPGRI). A search of the Bioversity database reveals that there are currently approximately 6,689 accessions of Kentucky bluegrass being maintained at 45 institutions worldwide. For all species of the *Poa* genus, there are approximately 8,383 accessions located at 64 institutions. Thus, Kentucky bluegrass represents 80% of all *Poa* germplasm accessions worldwide and over 90% of the accessions of the agronomically important *Poa* species listed in Table 1. Kentucky bluegrass also represents over 90% of all varieties listed by the Organisation for Economic Co-operation and Development (OECD, http://www.oecd.org/). As a whole, these germplasm resources primarily represent potential sources of parental material for performing crosses for the breeder, however, some of the natural ecotype accessions could potentially yield cultivars in and of themselves. Many of the Kentucky bluegrass accessions in germplasm repositories, either knowingly or unknowingly, represent commercialized cultivars. Thus, Kentucky bluegrass germplasm resources are likely under-represented, given its extensive genetic diversity and economic value. In addition, many of the minor bluegrass species are lacking entirely from these collections and so more effort needs to be given to collecting and managing bluegrass germplasm resources particularly for land restoration of native ecosystems (ex. Pickart 2008) and implementing land-use decisions (ex. Rudmann-Maurer et al. 2007).

Increasing the germplasm resources of bluegrasses and thereby increasingly the workload of repositories, given limited financial resources, is a difficult situation. Johnson et al. (1997) and Johnson et al. (2002) found that the majority of accessions within the extensive collection of Kentucky bluegrass maintained by the USDA/ARS at Pullman, WS actually represented only four major clusters based on agronomic characteristics (Johnston et al. 1997) and only one cluster of DNA marker diversity (Johnson et al. 2002). These researchers suggest that unique genotypes of Kentucky bluegrass are under-represented within the collection as a whole. In an effort to free up resources and to make room for additional collections, they were able to create a core collection group of 38 accessions to represent the majority of diversity within the total collection, at the time, of 348 accessions. This core group receives regular maintenance and increase while the remaining accessions receive more long-term storage conditions and less frequent increase. Wieners et al. (2006) further characterized the 38 accession core collection and found extensive diversity in terms of the level of apomixis and DNA content. Thus, creation

of core groups allows interested users a sampling of the total genetic diversity contained within the larger collection which can be followed by more detailed analyses of individual accessions in a hierarchical fashion saving both time and money. As proof that the additional efforts required to adequately characterize extensive bluegrass germplasm collections is warranted, the German plant breeders have helped fund an extensive characterization of European *Poa* collections using agronomic, molecular marker, cytology, and reproduction characteristics (Andreeva et al. 2003).

Such stratified management and detailed characterization of germplasm resources will likely become more common place because as human populations expand and as agricultural practices change, the in situ genetic resources of bluegrass germplasm become more and more at risk. The risk of land-use changes which threaten bluegrass genetic resources was the basis for van Treuren's (2008) research to investigate the Kentucky bluegrass genetic resources of "old" Dutch pastures. He compared AFLP fingerprints of Kentucky bluegrass plants collected from 17 naturalized grasslands and nature reserves to those of 11 commercial cultivars. He found that most populations contained a single, or only a few dominant genotypes, followed by numerous genotypes of low frequency and that there was considerable overlap in genetic diversity among natural grasslands. Eighty-eight percent of the grassland genotypes could not be ascribed to any of the references cultivars and were not part of the Netherland Centre for Genetic Resources collection suggesting, again, that Kentucky bluegrass diversity is under-represented within germplasm repositories.

5 Major Breeding Achievements

The first significant achievement for turfgrass improvement in Kentucky bluegrass came in the 1930s when Mr. Joseph Valentine, the then Superintendent of Merion Golf Club, Ardmore, PA, discovered a genotype of Kentucky bluegrass on one of the course's fairways that exhibited resistance to the disease leaf spot (*Drechslera poae* (Baudy Shoem). In collaboration with the United States Golf Association, the cultivar 'Merion' was developed from this genotype and, until the mid-1980s, was widely planted throughout the northeastern USA as lawn and golf turf (Alderson et al. 1995).

Probably the most significant impact in the forage industry was the development of the cultivar 'Kenblu' released in the 1950s. Kenblu was originally developed as a blend of different naturally occurring apomictic ecotypes that shared similar growth performances and phenologies (Alderson et al. 1995). DNA markers suggest that there are actually three main clades of genotypes which comprise the Kenblu cultivar (Huff 2001). Kenblu is still in use today for pastures and as a low maintenance turf which is perhaps a testament to the value of maintaining genetic diversity within a grass sward.

The most significant advancement for breeding new cultivars of Kentucky bluegrass came from the ability to make new genetic combinations through intraspecific hybridization. The commercial success of intraspecific hybridization was

dramatically illustrated by C. Reed Funk and his associates who developed a greenhouse crossing technique that resulted in an increased frequency of F1 hybrids compared to field crosses (Funk and Han 1967, Pepin and Funk 1971). Essentially, their technique was to allow plants to flower early in the spring under greenhouse conditions and to hand pollinate as early as possible after the stigmas emerge and are receptive (Bashaw and Funk 1987). Improvements to this technique were later made by Hintzen and van Wijk (1985) who used artificial lighting to promote long daylengths in order to further increase the frequency of Kentucky bluegrass hybridizations.

Another major development in the breeding of bluegrasses was contributed by numerous individuals in the area of interspecific hybridization (Muntzing 1940, Akerberg 1942, Akerberg and Bingefors 1953, Clausen et al. 1947, Clausen 1961, Clausen et al. 1962, Dale et al. 1975, van Dijk and Winkelhorst 1982). Jens Clausen and his associates at the Carnegie Institution of Washington, Stanford, CA, essentially summarized the goal of inter- and intraspecific hybridization efforts by Kentucky bluegrass breeders everywhere which is "to break the apomictic bond periodically releasing the variability and then sealing it up again after a period of recombination" (Clausen et al. 1947). The first commercial cultivar release involving interspecific hybridizations was contributed by James Read and his associates, Texas A&M, who developed the cultivar "Reveille" for turf use in the semi-arid regions of the southern USA. Reveille is an F1 hybrid between Kentucky bluegrass and Texas bluegrass (Read et al. 1999).

A milestone achievement for the evaluation of Kentucky bluegrass cultivars in the USA came in 1980 when J.J. Murray, USDA-ARS, orchestrated the first nationwide evaluation trial with 84 entries of Kentucky bluegrass being evaluated at 50 locations (Shearman 2006). The success of this trial eventually led to the formation of the National Turfgrass Evaluation Program (NTEP) which has since expanded its efforts to coordinate the evaluation of 17 turfgrass species in as many as 40 states in the USA and 6 provinces in Canada.

6 Specific Goals in Current Breeding

In general, there are many traits that make Kentucky bluegrass an agronomically important grass species (Wedin and Huff 1996). Kentucky bluegrass is a valuable pasture grass because it tolerates close and frequent grazing better than other cool-season forage grasses. Kentucky bluegrass is also one of the most popular turfgrasses because under regular mowing it produces a lush, dense turf possessing a green color that is pleasing to the eye.

Currently, the use of Kentucky bluegrass is becoming quite varied and specialized in both the forage and turfgrass industries. Thus, some of the specific goals for any Kentucky bluegrass breeding program will depend ultimately on the intended use of the resulting cultivars. Most varieties of Kentucky bluegrass have been developed for use as turf, however, there are specific goals for each of the areas of the

turfgrass industry. For example, the aggressive types of Kentucky bluegrass are intended specifically for use as athletic field turf in order to rapidly recover from wear and traffic. Adding even small percentages of aggressive types into lawn turf blends would not only increase the need for cultivation but would likely result in the dominance of the aggressive type within the lawn turf and thereby defeat the purpose of blending altogether. Cultivars intended for the lawn and landscape turf market may sometimes gain wider acceptance by possessing a more open (less dense) canopy (ex. cv. 'Moonlight'), thereby displaying less aggressiveness, to encourage the growth and persistence of other components within the home lawn mixture like perennial ryegrass or perhaps other bluegrasses. Other specific goals for breeding Kentucky bluegrass may be similar for either forage or turf markets. These goals would emphasize uniformity and stability of traits including disease and insect resistance, abiotic stress tolerance, persistence, seed quality and seed yield.

6.1 Disease Resistance

To a large extent, disease resistance has directed the utility of Kentucky bluegrass, particularly within the turfgrass industry, and thus is one of the most important goals breeders focus upon. Some of the major diseases affecting Kentucky bluegrass include leaf spot and melting out [*Drechslera poae* (Baudys) Shoem.], stem rust (*Puccinia graminis* Pers.), stripe rust (*Puccinia striiformis* f.sp. *Poae* Tollenaar & Houston), stripe smut [*Ustilago striiformis* (West.) Niessl.], summer patch (*Magnaporthe poae* Landschoot and Jackson), necrotic ring spot (*Leptosphaeria korrae* Walker and Smith), brown patch (*Rhizoctonia solani* J.G. Kühn), yellow patch (*Rhizoctonia cerealis* E.P. Hoeven), dollar spot (*Sclerotinia homoeocarpa* F.T. Benn), and powdery mildew [*Blumeria graminis* (DC.) Speer] (Smiley et al. 1992).

Variable host resistance for most of these diseases has been observed in Kentucky bluegrass. Thus, breeding for disease resistance requires an ability to either screen germplasm under natural sources of infection in the field or develop a methodology to culture the disease-causing organism and perform artificial inoculations. Most breeding programs simply allow natural infestations to occur and select for healthy plants. However, disease pressure in the field can be highly variable and non-uniform over space and time. On the other hand, rearing appropriate strains of disease-causing organisms and developing the experience to inoculate without overwhelming a plant's resistance mechanism requires a dedicated commitment of time and resources.

In a study by Czembor (2002) to screen for leaf spot-resistant germplasm from the Polish Gene Bank, artificial inoculations were compared to naturally occurring field infestations over a 3-year period. In addition, Czembor's study also compared the field resistance of Kentucky bluegrass ecotypes that had been previously screened and selected for leaf spot resistance under greenhouse conditions

(Czembor 2003). She found that, while artificial inoculation gave a more consistent and significantly higher level of disease severity compared to natural infestation, there was no discernable difference in plant resistance rankings between the two treatments. What was encouraging was the significant correlation between the greenhouse selection procedure and the artificially inoculated field results suggesting that screening for resistance to *D. poae* may be performed at any time throughout the year in the greenhouse.

Bonos et al. (2006) provide a detailed review of the progress made toward breeding Kentucky bluegrass for improved disease resistance to leaf spot and melting out, stem rust, and stripe smut. As Bonos et al. (2006) point out, the genetic resistance of Kentucky bluegrass cultivars to leaf spot has remained very stable over the years. For example, the cv. 'Merion' which was originally collected in the 1930s because of its resistance to leaf spot disease continued to display good field resistance throughout its commercial life which ended in the mid-1980s. 'Midnight' Kentucky bluegrass, released in 1984, is another fine example of stable genetic resistance to leaf spot and melting out disease.

Unfortunately, such genetic stability of host resistance over time is not a feature for all diseases affecting Kentucky bluegrass. 'Merion', the most widely planted cultivar of Kentucky bluegrass in the USA during the late 1940s and 1950s was by 1959 one of the most strip smut susceptible cultivars (Kreitlow and Juska 1959). In the northeastern USA, 'Merion' was eventually replaced as the preferred Kentucky bluegrass lawn turf, principally by the cultivar 'Baron' released in 1980 (Hurley and Ghysen 1980). In the early 1990s, C. Reed Funk, Rutgers University, observed that 'Baron', as well as all other cultivars genetically similar to 'Baron' (i.e., the BVMG types), was exhibiting stripe smut susceptibility. At the same time, 'Merion' was exhibiting relatively good tolerance to strip smut disease. Funk deduced that the disease-causing agent had shifted hosts, i.e., from Merion to Baron types (Funk pers. comm. 1992, Bonos et al. 2006). Bonos et al. (2006) provide an additional example of a potential host shift by the stripe smut disease citing the cultivar 'Shamrock' which has been exhibiting increased susceptibility to stripe smut since its release in the early 1990s.

Combating host shifts in disease-causing agents through conventional plant breeding is difficult enough in sexually reproducing crop species but it becomes even more of a challenge in asexual apomictic species which prevent breeders from simply backcrossing resistance genes into a susceptible commercial cultivar while retaining all of the commercial cultivar's favorable traits. This fact is particularly demonstrative in the case of breeding Kentucky bluegrass for stem rust resistance. Stem rust resistance in Kentucky bluegrass, as in other grasses, can be particularly short-lived. Cagas and Markova (1988) reported the development of new races of rust pathogens that caused outbreaks across Europe on Kentucky bluegrass. Stem rust resistance in Kentucky bluegrass is probably controlled by the action of a single gene (Bonos et al. 2006) yet commercial cultivars of Kentucky bluegrass which exhibit increased susceptibility over time, ex. Midnight, are unable to directly benefit from new sources of resistance without creating entirely new

cultivars. Thus, while research like that by Czembor et al. (2001) suggests resistance to rust diseases to be highly variable among a broad range of Polish ecotypes, breeders need to be aware that they will only be able to incorporate such new sources of resistance at the expense of recombining most, if not all, of their other traits.

6.2 Insect Resistance

Insect pests may also cause serious damage to Kentucky bluegrass pastures and turf. For example, numerous species of billbugs attack Kentucky bluegrass but the common types tend to exhibit more resistance than improved turf types (Kindler and Kinbacher 1975). Variable resistance to fall army worm (*Spodoptera frugiperda* Smith) forced feeding has been observed among Kentucky bluegrass cultivars and Texas bluegrass accessions but was found to be highly variable among Kentucky × Texas hybrids (Reinert and Read 2008). Genetically diverse cultivars of Kentucky bluegrass all appear susceptible to the greenbug (*Schizaphis graminum* Rondani) (Ratcliffe and Murray 1983). Kentucky bluegrass has known susceptibility to additional insects such as the winter grain mite (*Penthaleus major* Duges) and numerous grub species. These and other insects have been studied and screened to a lesser extent and so much more work is required for developing insect resistances in Kentucky bluegrass.

6.3 Environmental Stress Tolerance

Of all the environmental stresses that impact Kentucky bluegrass performance and persistence, the most debilitating are heat, drought, and salinity. Heat and drought tolerance among cultivars of Kentucky bluegrass tends to be moderate to low and restricts the broader use of the species. Bonos and Murphy (1999) examined a range of Kentucky bluegrasses for a combination of heat and drought stress and found that tolerance was attributable to a greater ability for water uptake at deeper soil depths. However, Richardson et al. (2008) specifically investigated drought tolerance among 50 Kentucky bluegrass turfgrass entries using digital image analysis and, although they found a wide range of variability, they found no correlation with rooting depth or root:shoot ratio characteristics. He and Huang (2007) examined specifically for heat tolerance and found that maintenance of protein stability was a main factor for heat tolerance in Kentucky bluegrass.

Improving heat and drought tolerance in Kentucky bluegrass through interspecific hybridization with Texas bluegrass, a native of southwestern USA, is also being investigated. Currently, however, there seems to be mixed results for the improved tolerances of these hybrids. In growth chamber studies, Abraham et al. (2004) observed considerable variation in drought tolerance among 30 hybrids and

their genetic parents and Abraham et al. (2008) found that two experimental hybrids exhibited improved heat and drought tolerance compared to two Kentucky bluegrass cultivars. However, Bremer et al. (2006), in a field experiment, found no differences in drought tolerance between two hybrids ('Thermal Blue' and 'Dura Blue') and 'Apollo' Kentucky bluegrass. In another growth chamber experiment, Su et al. (2007) found that Thermal Blue hybrid was more heat tolerant than Apollo Kentucky bluegrass and 'Dynasty' tall fescue but no differences were observed for drought tolerance. Given the extensive genetic variability within the available Kentucky bluegrass parental germplasm coupled with the extreme segregation patterns of aberrant hybrid progenies, it seems only reasonable that it will take time to sort out the appropriate epistatic gene combinations to successfully develop improved heat and drought tolerances in these interspecific hybrids.

Kentucky bluegrass notably lacks salt tolerance and is generally considered to be a salt-sensitive species (Carrow and Duncan 1998). However, Suplick-Ploense et al. (2002) observed a broad range of salt tolerance variability in terms of leaf firing and shoot and root growth reduction among nine Kentucky bluegrass cultivars, three Texas bluegrass accessions, and five Kentucky × Texas hybrids. Thus, potential for improving salt tolerance in Kentucky bluegrass may exist. In addition, moderate levels of salt tolerance have recently been shown to exist in annual bluegrass that are as high or even higher than some creeping bentgrasses (*Agrostis stolonifera*) (Dai et al. 2008, 2009) and so perhaps other *Poa* species may be capable of providing the needed levels of salt tolerance.

6.4 Forage Yield

Assessment of forage yield is an important component of VCU testing schemes for cultivar registration and recommendation. The German descriptive list of fodder grasses (Bundessortenamt 2007) shows large variation in forage yield potential of cultivars, with tall-growing and broad-leaved 'Lato' setting an overall yield benchmark which has not been topped since it's registration in 1989. Among the cultivars listed in Germany for forage use, 'Lato' is the only sexual cultivar, (Freudenstein, pers. comm.) suggesting that it may be easier to select for high forage yield in sexually propagating populations.

Dürr et al. (2005) examined herbage yield for six Kentucky bluegrass cultivars originating from Norway, Germany, and North America grown under two harvest systems at two locations in eastern Canada. They found that all cultivars persisted over the 3 years of the production study, dry matter yields of Kentucky bluegrasses were similar to those of timothy, and that warm and dry conditions favored the narrow-leaved Kentucky bluegrasses (presumably subsp. *angustifolia*). For forage purposes, hybrids between Kentucky bluegrass and Sandberg bluegrass are being made and evaluated for the appropriate combination of forage production and heat tolerance as are Texas bluegrass hybrids with other *Poa* species (Kindiger 2004, Goldman and Sims 2005).

7 Breeding Methods and Specific Techniques

Kentucky bluegrass typically displays a wealth of genetic diversity within its available germplasm resources. In addition, these germplasm resources contain many desirable characteristics for turf and forage utility. However, breeders are unable to utilize methods of recurrent selection and backcrossing to recombine these desirable traits together into one cultivar or one interbreeding population in the presence of the asexual apomictic breeding system of Kentucky bluegrass (Funk 2000). Thus, the feature that makes breeding Kentucky bluegrass so different from most other grasses is its facultative apomixis mode of reproduction.

Assessing the level of apomixis is important for the release of Kentucky bluegrass cultivars because it determines the uniformity and stability of the cultivar across generations of seed production. Most breeding lines of Kentucky bluegrass released as cultivars typically express high levels of apomixis, 95% or more. A similarly high level of apomixis is found for most germplasm accessions occurring in nature (Mazzucato et al. 1996). However, some lines with as low as 80% apomixis have also been released as seeded cultivars because the aberrant off-type plants produced (mostly polyhaploids and BIII selves) tend to be weak and are believed to be outcompeted during the establishment phase (Figure 3; also see Table 2). Cultivars with as low as 20% apomixis may also find their way into the commercial market place, however, these lines become vegetatively propagated cultivars for sod farms such as 'Warren's A-20'. There have also been a few cultivars released as purely sexual populations of Kentucky bluegrass, for example, 'Lato' and 'Jori' (see Matzk et al. 2005).

Fig. 3 Three progeny plants grown from polyembryonic seed of cv. "Baron" Kentucky bluegrass that were derived from different genetic pathways of apomixis reproduction. Pseudogamous development of an unreduced aposporous egg yields a typical apomictic Baron plant (*center*). Pseudogamous development of a reduced meiotic egg yields a polyhaploid plant with approximately $^1/_2$ the DNA of a typical "Baron" plant (*left*). Pollen fertilization of an unreduced aposporous egg yields a BIII hybrid plant with approximately 1.5 times the amount of DNA of a typical "Baron" plant (*right*) (Photo D. Huff)

When applying for plant breeders' rights or cultivar registration involving DUS testing, breeders have to declare the mode of reproduction of their candidate cultivar. The testing procedures and requirements for homogeneity of monoclonal apomictic cultivars are different from those of non-apomictic cultivars. Only two aberrant plants out of 30 planted are tolerated in monoclonal apomictic cultivars, while each DUS characteristic of non-apomictic cultivars may vary across a range set by the observation of comparable previously registered cultivars.

Various methods are available to determine the level of apomixis in Kentucky bluegrass. The most sophisticated screening method was recently developed by Matzk et al. (2000) and is known as the flow cytometric seed screen (FCSS). FCSS utilizes the ploidy relationships between the embryo and endosperm within a seed to discriminate the genetic origin of eggs and pollen derived through either the apomictic or sexual pathway. Moreover, the efficiency of FCSS may be increased by examining two seeds at the same time because the sensitivity of flow cytometry enables the detection of two embryo and/or endosperm peaks derived through different pathways. FCSS is such a powerful tool that it is also capable of distinguishing the components of the overall apomixis pathway in Kentucky bluegrass, namely apospory and parthenogensis (Matzk et al. 2000) (see Table 2). Thus, FCSS is not only beneficial to breeders for screening apomixis levels but aids researchers in unraveling the genetic basis of apomixis.

Another technique useful for closely approximating the frequency of apomixis, particularly when flow cytometers are inaccessible, is the "auxin test" also developed by Matzk (1991b). In the auxin test, the capacity for the parthenogenic seed development component of apomixis is assessed by dipping inflorescences into a 100 ppm synthetic auxin solution of 2,4-dichlorophenoxy acetic acid (2,4-D) a day or two before anthesis. Approximately 2 weeks after anthesis, the parthenogenic frequency is determined as the percentage of those caryopses containing one or more differentiated embryos using a dissecting microscope. Barcaccia et al. (1998) used the auxin test to confirm the monogenic inheritance of parthenogenesis in controlled crosses of Kentucky bluegrass. Szabó and Papp (2005) utilized the auxin test to survey the level of parthenogenesis in six populations of each of three subordinate taxa of Kentucky bluegrass: *P. pratensis* L. subsp. *pratensis*, *Poa angustifolia* L. [= *P. pratensis* L. subsp. *angustifolia* (L.) Dumort.], and *Poa humilis* Ehrh. ex Hoffm. [= *P. pratensis* subsp. *irrigata* (Lindm.) H. Lindb.]. A wide range of variation was found among populations within taxa but no significant differences were observed between taxa although *P. humulis* did exhibit the least overall parthenogenesis.

Another method for determining the level of apomixis is by direct examination of the ovule embryo sacs. Determining the embryonic constitution of ovules is accomplished by the pistil clearing technique which involves dehydration of pistils in an ethanol series followed by treatment in a clearing media making cells transparent and amenable to phase-contrast microscopy (Young et al. 1979). Though extremely laborious and time consuming, the pistil clearing technique is arguably the most satisfying for many apomictic plant species because each individual ovule is

visually inspected. However, in Kentucky bluegrass, unreduced aposporous embryo sacs typically contain 8-nuclei making it very difficult to distinguish from the 8-nuclei reduced meiotic embryo sac (Huff pers. obs.). Thus, the other techniques mentioned previously are more useful for Kentucky bluegrass.

The most common method for determining the level of apomixis within Kentucky bluegrass breeding program continues to be the visual inspection of progeny plants grown in a spaced-plant nursery. For the spaced-plant progeny test method, progeny plants derived from single seeds collected from an individual seed-bearing parent are grown in the greenhouse and then transplanted to the field in a series of rows representing various experimental lines or accessions. Seed-bearing parents exhibiting high levels of apomixis display progeny plants that are uniform in appearance and phenology and which resemble the parent plant. However, some maternal parents will produce "off-type" progeny plants which are distinctly different in appearance and/or phenology compared to the parent from which they were derived. The level of apomixis of any particular maternal parent is determined by the frequency of progeny plants which are off-types (also known as aberrants). These off-type aberrant progeny represent new genetic combinations and thus are potentially new cultivars. Unfortunately, most aberrant plants lack the specific combination of traits desired in an improved cultivar and so many aberrant progenies need to be evaluated in order to identify a superior genotype.

Breeding an apomictic species can lead to cultivar release more rapidly than breeding a sexually reproducing species. This is because segregation does not occur in highly apomictic plants and thus the breeder immediately attains genetic uniformity and stability once the superior genotype has been identified. Thus, new selections from old pastures or old turfs that possess all the desirable traits and high levels of apomixis are capable of directly becoming new cultivars. A good example is the selection made by Mr. Joseph Valentine, who was not a trained plant breeder, and yet was able to contribute a significant advancement toward Kentucky bluegrass improvement simply by selecting a naturally occurring genotype on his golf course. Ecotypic selections like Mr. Valentine's have played a large role in Kentucky bluegrass improvement, however, there are limitations to this type of breeding.

7.1 Ecotype Selections

Rich germplasm resources of bluegrasses may be found in old pastures, meadows, and turfs. Some of these genotypes have persisted over long periods of time attesting to their inherent ability to be competitive against other grasses and to withstand environmental extremes and disease and insect pressure. Genetic diversity among Kentucky bluegrass genotypes has not been found to be related to geographic distribution at a large scale (Johnson et al. 2002, Andreeva et al. 2003) or on a small scale (van Treuren 2008) and thus diversity is not necessarily predictable based on geographic location. This means that potentially valuable collection sites may as easily

exist locally as compared to other more exotic locations. The problem is that many of the potential collection sites which are easily accessible may have already been accessed by previous breeders, and as such, today's Kentucky bluegrass breeders are often expanding their collection efforts into more and more inaccessible regions of the world, ex. Mongolia or isolated valleys of Pennsylvania and central Europe.

As a general rule, North American grass breeders will typically collect germplasm from more northern latitudes and more western longitudes than the intended area of use to gain increased tolerance to cold temperatures and drought; or conversely, from more southern latitudes and more eastern longitudes for increased tolerance to heat, humidity, and disease pressure. Kentucky bluegrass genotypes have been shown to exhibit site-specific adaptability (Annicchiarico et al. 2006) as well as circumpolar distribution (Soreng 1990) and thus, few predictions regarding geographic origin and associated traits are possible. The most common strategy is to collect as much germplasm as you can, from as many sites as possible, and to perform extensive evaluations under extreme conditions. A notable success resulting from this very strategy is the cultivar "Unique" (Rose-Fricker et al. 1999).

Prior to 1970, all cultivars of Kentucky bluegrass were initially collected from pre-existing naturalized grasslands or cultivated turfs. Today, however, most cultivars are derived from intraspecific hybridizations.

7.2 Intraspecific Hybridizations

Two strategies exist for performing intraspecific hybridizations in Kentucky bluegrass. The more common of these strategies is to make pairwise crosses between parental genotypes that are each highly apomicitic. The advantage of this strategy is that any resulting aberrant progeny tend to also exhibit high levels of apomixis and therefore have the potential of directly entering into the market place after extensive performance evaluation. The disadvantage of this strategy is that the frequency of aberrant progeny produced from such crosses is inherently low due to the high levels of apomixis of the maternal parent. To overcome this disadvantage, large numbers of progenies must be evaluated for their aberrant status by either visual inspection, or otherwise screened using molecular markers or flow cytometry or some combination of the three. In addition, crossing techniques have been discovered over the years that significantly increase the number of aberrant offspring derived from a highly apomictic female parent. One such technique is to transfer field grown vernalized plants into a greenhouse (typically late winter/early spring) and allow these plants to flower under artificial long daylength conditions and then to pollinate as early as possible after the stigmas emerge and are receptive (Funk and Han 1967, Hintzen and van Wijk 1985). Pollinating immediately as the maternal parent flowers open has been suggested to increase chances of fertilizing eggs (either sexual or aposporous) (Bashaw and Funk 1987) because the apomictic proembryo often begins development at or slightly before anthesis (Akerberg and Bingefors 1953). Pepin and Funk (1971) found that these early pollinations resulted in high numbers

of BIII intraspecific hybrids as compared to other possible genetic outcomes (see Table 2).

Pollinations may be performed by hand, but because Kentucky bluegrass flowers tend to open between 1:00 and 4:00 am under greenhouse conditions (Funk and Han 1967), many breeders utilize mechanical means for effecting pollinations (Figure 4). Several methods of mechanical pollination have been described to facilitate hybridization in Kentucky bluegrass (Hintzen and van Wijk 1985, Riordan et al. 1988). The method depicted in Figure 4 uses a wooden framed wire rack suspended from ceiling chains to allow movement when nudged by a small electric motor running on a timer set to produce three nudges a minute every 3 minutes. Inflorescences are bagged in a ratio of five male inflorescences to one female inflorescence to ensure pairwise crossing. The pollination bags are then attached to the wire rack using wooden stakes and large paper clips and secured around the inflorescences at the base of the bag with large paper clips. The advantages of this particular method are that it allows for a large number of pairwise crosses to be performed in a relatively small space and that height differences between parent plant inflorescences are easily accommodated by stacking different numbers of upside down clay pots as a base. In addition, multiple racks may be attached to one another to further increase the number of crosses performed (depicted in Figure 4).

While the frequency of aberrant progeny derived from an intraspecific cross mostly depends on the choice of female seed-bearing parent (Hintzen 1979), small influences from the male pollen parent have been detected in the frequency of aberrants (Gates 1997) and in seed set (Grazi et al. 1961). In addition, the agronomic performance of hybrid aberrant progeny often depends on both the male and female parents (Hintzen 1979, Gates 1997).

The other strategy for making intraspecific hybridizations in Kentucky bluegrass is to use parent plants (typically one or sometimes both) that are highly sexual (non-aposporic and non-parthenogenic). The advantages of this strategy are that

Fig. 4 A mechanical crossing apparatus used to facilitate intra- and interspecific hybridizations in Kentucky bluegrass (see text for details) (Photo D. Huff)

an increased frequency of aberrant progeny will be produced giving the breeder a maximum number of recombinant progeny from which to perform selection and that the breeder will have higher confidence that any off-type plants produced will be genetically aberrant progeny. However, the disadvantage here is that levels of apomixis among the progeny will tend to be low. To overcome this disadvantage, large numbers of progeny need to be evaluated, in much the same fashion as the strategy employing highly apomicitic parents, in order to identify progeny which possess high levels of apomixis. Most often, high levels of apomixis are not frequently observed in the F1 progeny of such crosses but rather in the succeeding F2 and F3 generations (Huff pers. obs.; see Akerberg and Bingefors 1953) as the genetic elements controlling apomixis recombine. However, depending upon the involved parents, highly apomicitic F1 hybrids have been recovered from such sexual × apomictic crosses by Matzk (1991a) who, as a result, has proposed a scheme of recurrent hybridization breeding for Kentucky bluegrass. As such, this particular strategy of interspecific hybridization offers great potential and deserves further investigation.

7.3 Interspecific Hybridizations

In much the same way that the retention of pollen recognition systems, coupled with the ability of apomixis to propagate non-homologous euploid and aneuploid genomes, has enabled the phylogenetic reticulation of bluegrass genomic mixtures to have occurred within the *Poa* genus, so too have these same elements allowed breeders to explore a broad spectrum of interspecific hybridizations within the bluegrasses. Many different examples of artificial interspecific hybridization exist in the literature, including: Kentucky bluegrass × Canada bluegrass (Dale et al. 1975); Kentucky bluegrass × Alpine bluegrass (Akerberg 1942, Akerberg and Bingefors 1953); Sandberg bluegrass × Kentucky bluegrass (Clausen et al. 1947, Hiesey and Nobs 1982); *P. longifolia* × Kentucky bluegrass (Almgard 1966, van Dijk and Winklehorst 1982), Texas bluegrass × Kentucky bluegrass (Read et al. 1999); and, Texas bluegrass × Argentine bluegrass (*P. ligularis* Nees. Ap. Steudel) (Goldman and Sims 2005). Such interspecific hybridizations offer potential sources of novel variation for both breeders and apomictic researchers. For example, Kindiger (2004) found that crosses between Texas bluegrass as the female parent and Sandberg (Big) bluegrass as the pollen source produced a low frequency of androgenic polyhaploid progeny plants containing only the DNA of Sandberg bluegrass.

However, despite the wealth of variability derived from interspecific hybridization, few of them have resulted in commercial cultivars. Bashaw and Funk (1987) believed that it is the long-term commitment required for such interspecific hybridization breeding strategies that explained the lack of commercial success. Part of this long-term commitment results from the loss of apomixis in some of these hybridization combinations as observed by Muntzing (1940) and the continued

evaluation of succeeding generations required for recovering apomictic recombinants as detected by Akerberg and Bingefors (1953). DNA markers are now frequently used by bluegrass breeders and researchers to facilitate detection of interspecific hybridization (Kindiger 2006, Goldman 2008).

7.4 Polyembryonic Seed

Another potential source of genetic variability for Kentucky bluegrass breeders to consider derives from polyembryonic seed (Andersen 1927, Akerberg 1939, Nielsen 1946, Duich and Musser 1959) (see Figure 3). Recently, a programmed cell death mechanism has been discovered which prevents the development of polyembryonic seed in higher plants (Filonova et al. 2002); however, polyembryonic seed is commonly observed in apomictic Kentucky bluegrass with levels reaching as high as 30% for some cultivars (Huff 1997). If properly cared for, the multiple seedlings arising from a single seed will produce an array of non-identical twin, triplet, and quadruplet seedlings. Many of these seedlings result from different genetic origins of the apomictic pathway and as such represent a potential source of aberrant progeny (Figure 3). For example, Wieners et al. (2006) found that 4 out of 38 Kentucky bluegrass accessions exhibited evidence for polyhaploid embryos while Huff and his associates (Huff and Bara 1993, Huff 1997) found that nearly half of all twin seedlings from Kentucky bluegrass cultivar ‘Baron’ were polyhaploid based on flow cytometry data. Moreover, Ostazeski et al. (1975) found that plants derived from polyembryonic seed provided new sources of resistance to leaf spot and powdery mildew diseases when compared to the seed-bearing parent. Thus, the potential for polyembryonic seed to be a useful tool for apomixis research and breeding in bluegrass species should not be overlooked.

8 Integration of New Biotechnologies

Apomixis is a brilliant means of fixing heterotic gene and genomic combinations, however, at the same time, apomixis inhibits breeders from accessing recombination and segregation in order to create new gene combinations. Furthermore, the complexities of the Kentucky bluegrass genome, which has become amalgamated under the auspices of apomixis, ensure that when segregation does occur there will be very little predictive power in terms of agronomic trait inheritance. Therefore, breeders will benefit from any biotechnological advances that give any amount of predictability or other assistance in harnessing apomictic reproduction.

Matzk et al. (2005) performed an extensive FCSS screening experiment on a series of segregating populations constructed through crosses and self-pollinations and developed an elaborate model for the genetic control of apomixis in Kentucky bluegrass. They concluded that at least five major genes were involved, namely, the apospory initiator (Ait) gene, the apospory preventer (Apv) gene, a

megaspore development (Mdv) gene, the parthenogenesis initiator (Pit) gene, and the parthenogenesis preventer (Ppv) gene. These predicted genes, along with their interactions, were capable of explaining the segregation patterns among the numerous segregating populations under investigation (Matzk et al. 2005).

The construction of genetic linkage maps is difficult in apomictic species like Kentucky bluegrass due to high levels of polyploidy and aneuploidy and the subsequent effects these genomic states have upon marker segregation. However, Porceddu et al. (2002) provide an example of linkage mapping in Kentucky bluegrass which directly addresses these unavoidable problems. Their results suggest that parental-specific single dose markers were useful for constructing linkage maps of either the apomictic or sexual parents and that at least some Kentucky bluegrass chromosomes pair preferentially during meiosis suggesting that Kentucky bluegrass is at least part allopolyploidy (Porceddu et al. 2002).

Albertini et al. (2004, 2005) utilizing the AFLP transcriptional profiling technique within a segregating population between a purely sexual line (non-aposporic and non-parthenogenic) and a highly apomictic line (aposporic and parthenogenic) were able to isolate and characterize two genes linked to the apomicitic pathway in Kentucky bluegrass: SERK (SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE) and APOSTART, named for the regulatory START domain which it contains and for its putative role in apomixis. In *Arabidopsis*, the SERK gene is involved in the embryogenic competence from somatic cells (Chaudhury and Peacock 1993) while the function of APOSTART is unknown though it resembles a MB1-like gene potentially involved in 2*n* egg formation (Barcaccia et al. 2001). Albertini et al. (2005) found eight polymorphic forms of SERK and nine polymorphic forms of APOSTART using Southern blot hybridization analysis. These researchers were able to demonstrate that different forms of reproduction (apomictic, sexual, or recombinant) exhibited different patterns of SERK polymorphisms and different expression profiles for two of the Kentucky bluegrass SERK alleles (PpSERK1 and PpSERK2). These differences are providing valuable insight into the underlying genetic mechanism of apomixis but have not, as yet, had immediate application for the breeder. APOSTART, however, is beginning to see some application in bluegrass breeding programs as a marker for apomixis. Albertini et al. (2005) were able to clone two allele-specific forms of APOSTART from Kentucky bluegrass (APOSTART1 and APOSTART2). Both APOSTART1 and APOSTART2 were expressed exclusively in inflorescences. However, expression of APOSTART1 is nearly lacking in apomictic plants. Albertini et al. (2005) hypothesize that APOSTART1 is involved in sporogenesis and thus its presence is an indication of non-apomictic reproduction. Use of APOSTART1 as a marker for apomixis may enable breeders the ability to screen parent and/or progeny plants more accurately and at an earlier time (first inflorescence production) than would be possible using the spaced-plant progeny test nursery technique. Time will tell if this potential will become widely utilized.

Unraveling of the complex genomic structure of apomictic bluegrasses and understanding of how its constituents interact would potentially benefit breeders throughout the breeding process. For example, one effect of interacting genomes

is the release and increased activity of transposable genetic elements as well as epigenetic silencing mechanisms (Matzke and Matzke 1998, Hegarty and Hiscock 2008). In annual bluegrass, evidence is beginning to accumulate which indicates that epigenetic silencing has a profound effect on life history characteristics including seed yield, vegetative growth, and persistence (La Mantia 2009). The most likely cause of epigenetic regulation in annual bluegrass is from its allopolyploid evolutionary history. Evidence for a similar allopolyploid evolutionary history has been proposed for Kentucky bluegrass as well (Grun 1955, Porceddu et al. 2002, Patterson et al. 2005). With the number of diploid genomes comprising Kentucky bluegrass, it seems reasonable to expect that epigenetic regulation might also be affecting similar traits including perhaps the trait of apomixis itself. Such epigenetic regulation might even play a partial role in the duplicate-gene asynchrony model proposed by Carman (1997) which contends that apomixis is caused by the "interference" of developmental pathways that result from hybridization of divergent genetic backgrounds.

Tissue culture also has a role in the development of Kentucky bluegrass cultivars. Generation of somaclonal variation is one potential application of tissue culture techniques toward Kentucky bluegrass cultivar development. Another application of tissue culture is during the transformation process of gene insertion. Researchers investigating the use of tissue culture in Kentucky bluegrass have successfully regenerated whole plants using several different sources of explants including mature embryos from seed (McDonnell and Conger 1984, Wu and Jampates 1986, Griffin and Dibble 1995, Ke and Lee 1996, van der Valk et al. 1989, Stephens et al. 2006), inflorescence tissue (van der Valk et al. 1989, Stephens et al. 2006), suspension culture (Nielsen and Knudsen 1993), and protoplast cultures (Nielsen et al. 1993). As is typical with culture technique, the frequency of regenerates obtained will depend on the genotype under investigation (see van der Valk et al. 1995) but recently even the frequency of apomictic and aberrant plants regenerated has been found to be dependent on the source of explants used (Stephens et al. 2006). Progress continues to be made in the area of tissue culturing Kentucky bluegrass for the potential generation of somaclonal variation and eventually the genetic transformation of the species (Hu et al. 2005).

Genetic transformation of crop plants may complement breeding efforts to combat problems caused by human population pressures and changing climates. This applies to forage and turfgrass as well as where water conservation and reduced pesticide and fertilizer inputs have desirable economic and environmental benefits. Although few bluegrass species have been evaluated for their potential to be genetically transformed, the most serious efforts have been directed toward Kentucky bluegrass. High frequencies of transgenic Kentucky bluegrasses have been reported by Gao et al. (2006) from the biolistic bombardment of embryonic calli derived from immature embryos. Gao et al. (2006) found that 34–78% of either bialaphos or hygromycin-resistant plants, respectively, were of independent origin. They also revealed a complex integration pattern of transgene inserts which perhaps suggests a measure of flexibility in Kentucky bluegrass genomic organization and hence gene insertion. Furthermore, Ha et al. (2001) were capable of confirming the stable

integration of three transgenes into the Kentucky bluegrass cultivar "Kenblu" and found coexpression frequencies of 20% for all three transgenes and 30–40% for two of the three transgenes.

Gene flow is a fact of nature particularly among plants which are rooted in the ground and release their gametes for the wind to spread. And although selfing and apomictic forms of reproduction may indeed limit gene flow, they certainly do not prevent it. Even highly apomictic plants can effectively pollinate adjacent plants possessing sexually outcrossing modes of reproduction whether they are of the same or different species. In a gene flow experiment performed under field conditions, Johnson et al. (2006) demonstrated that highly apomictic pollen donor plants of a transgenic Kentucky bluegrass were capable of effecting hybridization in five out of six apomictic Kentucky bluegrass genotypes, in both *Poa secunda* × *pratensis* hybrid accessions examined, and in one accession of Texas bluegrass. Although the frequencies of hybrids were small (ranging from 0.02% in one of the Kentucky bluegrass genotypes to 3.4% in the Texas bluegrass accession) and no hybrids were recovered from any of the remaining 29 accessions representing 23 species, nevertheless, this study demonstrates that apomixis does not eliminate gene flow and thus assessments will be required to determine the extent and potential for environmental impact of transgenic bluegrasses.

The integration of new biotechnologies is just beginning to be applied to the development of Kentucky bluegrass cultivars and will only increase as time goes on. In addition, many of these technologies will find applications in other parts of the commercial industry as well. For example, the value of genetic markers in the Kentucky bluegrass seed processing and sod production industries is currently being considered for improving quality assurance and quality control.

9 Seed Production

Currently, the main areas of Kentucky bluegrass seed production in the world are the Pacific Northwest USA (Washington, Idaho, and Oregon), northern Minnesota, Canada, Denmark, and the Netherlands. Most seed production is focused on proprietary cultivars and inputs of fertilizers and pesticides as needed to maximize yields of high-quality seed. In order to reduce lodging and concentrate inflorescences for harvest, seed fields of Kentucky bluegrass are often sprayed with a growth-regulating herbicide such as trinexapacethyl [4-(cyclopropyl-*a*-hydroxymethylene)-3,5-dioxocyclohexanecarboxylic acid ethylester] or prohexadione calcium (calcium 3-oxido-5-oxo-4-propionylcyclohex-3-enecarboxylate) (Holman et al. 2007).

In the past, Kentucky bluegrass seed production in the Pacific northwestern USA and in Denmark was based on an agricultural technique of burning away the straw stubble after seed harvest was complete. Burning straw and stubble residue improved seed yield to a greater extent than mechanical removal and also provided a measure of disease control as well as reinvigorating the stand to extend the number of production seasons (Canode and Law 1979, Steiner et al. 2006, Holman

et al. 2007). Unfortunately, the smoke produced during the act of burning seed fields created serious local air quality issues and, on occasion, fatal conditions along adjacent roadways and interstate highways due to poor visibility. As a result, the practice of burning grass seed fields is being phased out and replacement agricultural practices are currently being sought. One potential strategy to replace the practice of burning would be to breed bluegrass cultivars that yield well, year after year, in the absence of burning or perhaps yield a secondary crop of straw useful for other purposes including biofuels or as a building material component. Another potential solution would be to utilize the straw as forage after seed harvest was complete. However, most cultivars under commercial production are turfgrass cultivars whose forage yields and nutrient composition are unknown. Holman et al. (2007) investigated various components influencing forage yield and nutrient composition and suggested that "selectively breeding for (cultivars with) less structural carbohydrate concentration or greater nonstructural carbohydrate concentration in tall cultivars might result in a cultivar with high forage yield and improved nutrient level".

General observations among breeders suggest that a negative correlation exists between turf or forage quality and seed yield in Kentucky bluegrass. In other words, selection for increased vegetative shoot density to improve turf quality or increase herbage yield tends to result in plants of lower seed yield. One possible explanation for this negative correlation might be a result of indirect selection for increased perenniality and thereby decreasing meristem determinacy keeping shoot apical meristems in a vegetative state and inhibit flowering. Certainly more research is needed in this area for Kentucky bluegrass as greater resource allocations toward vegetative growth are desirable for both the forage and turfgrass markets. Because no matter how attractive or productive a plant or population may be, the need for economically sustainable seed yield is a perquisite for cultivar release unless the sole method of commercialization is through vegetative propagation. Thus, most Kentucky bluegrass breeders find that they must balance their selection between vegetative growth characteristics and seed yield potential. Occasionally, this negative correlation is found to be weak or lacking and, when this occurs, the breeder has great potential for success.

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Minor Grass Species

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1 Introduction

Besides the five major grass genera used widely for both agriculture and non-agriculture purposes, there is quite a large group of neglected cool-season minor grass species which enrich the crop spectrum. The term 'minor grasses' refers to the degree of attention paid to these species by scientists, plant breeders, germplasm conservationists, and the commercial sector. The mentioned species are also classed as 'secondary' in their importance to agriculture (Spedding and Diekmahns 1972). Minor grass species are commonly found in different types of natural, semi-natural, and sown grassland, such as permanent pastures and meadows, lawns in which they are adapted to a wide range of environmental factors. They are often dominant and/or diagnostic species and their names are associated with vegetation units (e.g., *Arrhenatherion elatioris*, *Cynosurion cristati,* and *Trisetion flavescentis*).

Despite these facts, they have been bred for special purposes such as specific environmental conditions and usage. Some of the minor grasses are traditionally cultivated for forage (e.g., brome grasses, meadow foxtail, reed canarygrass, tall and golden oat grass), mainly in mixed stands. Others play an important role in the turf and gardening industry, such as bentgrasses in golf courses, or are planted for soil protection or erosion control. In the past decades the importance of minor grasses has increased in response to the public interest in restoration of disturbed landscapes by 'near-natural' methods of re-vegetation. Minor grasses are added as important components to site-specific species-rich seed mixtures to increase plant species diversity of newly established grassland. The need for species diversification has resulted in new varieties of 'wild' grass species developed for landscaping and turfs (e.g., *Anthoxanthum odoratum* and *Koeleria macrantha*).

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2 Origin and Systematics

Cool-season minor grasses are widely naturalized in temperate zones throughout the world. According to Tutin et al. (1980) minor grass species belong to following tribes: Poeae (*Cynosurus cristatus*, *Puccinellia distans*, *Bromus* spp.), Aveneae (*Holcus lanatus, Deschampsia cespitosa, Trisetum flavescens, Arrhenatherum elatius, K. macrantha*), Agrostideae (*Alopecurus pratensis* and *Agrostis* sp.), and Phalarideae (*A. odoratum* and *Phalaris arundinacea*). Major systematic problems in this group of species concern the genus *Bromus* within the section *Ceratochloa* which contains a number of perennial and annual species from South America, Africa, and western North America. Many of these species are variable, often difficult to identify, and have been mentioned in various publications under different taxonomic names (Rumball and Forde 1976 in Stewart 1996). For more details see species description.

3 Varietal Groups

The number of bred varieties is relatively low in comparison to major grass species (see OECD list of varieties 2009, http://www.oecd.org/dataoecd/52/16/41920785.pdf). Most attention has been paid to bromegrasses and bentgrasses. In some species only a few varieties were developed in the world (i.e., *A. odoratum, P. distans, Koeleria cristata*). According to their usage, minor grasses comprise forage as well as amenity grasses and also forms used for horticulture purpose. Decorative plant types of attractive color of leaves or inflorescence have been developed in *A. pratensis*, *A. elatius* ssp. *bulbosum*, *Bromus inermis*, *D. cespitosa,* and *P. arundinacea*. They must be propagated vegetatively to retain their typical morphological characteristics.

4 Genetic Resources and Utilization

There are no large germplasm collections of minor crop plants including grasses in European gene banks and institutions (Hammer and Spahillari 2000). For the best represented genus among minor grasses, *Agrostis*, 1615 accessions have been maintained in comparison with 11228 accessions of the genus *Lolium* in ex situ plant collections in Europe (EURISCO 2009). In the USA, the highest number of accessions in the germplasm collection among minor grasses is reported for the genus *Bromus* (GRIN 2009). Wide interest in *Bromus* is also indicated by the large number of accessions in the New Zealand and Australian germplasm collections (Williams 1996; Margot Forde Germ. Cent. 2009) (Table 1).

The predominant sample status of germplasm accessions is wild. Landraces of minor grasses are usually not available and have already disappeared, if there were any. In many cases the germplasm of the oldest varieties have also been lost after

Table 1 Minor grasses germplasm ex situ collections

Genus	EURISCO (Europe)	GRIN (USA)	Margot Forde Germplasm Centre (New Zealand)
Agrostis	1615	238	568
Alopecurus	419	148	9
Anthoxanthum	70	5	122
Arrhenatherum	385	140	175
Bromus	1193	1006	1180
Cynosurus	155	29	292
Deschampsia	406	119	9
Holcus	128	21	243
Koeleria	94	129	20
Phalaris	369	715	572
Puccinellia	38	36	7
Trisetum	70	21	16

they were replaced by new cultivars or the grass species was withdrawn from previous cultivation as a crop (e.g., *P. arundinacea* in Germany). However, for the majority of minor grass species, wild populations still widely exist in natural habitats and thus can be collected to preserve their genetic diversity for present and future utilization. Currently, the European Central Crop Database for Minor Forage Grasses is being developed by NordGen, Alnarp, Sweden.

5 Species

5.1 *Agrostis canina* L. ssp. *canina* – Velvet Bentgrass, Velvet Bent

Native to Europe and temperate Asia. Limited to wet and very wet soils. Suitable only for turf purposes, introduced to USA in early 1900s as a component of seed mixtures sold as South German bentgrass. Velvet bent has been recognized for many years as forming the most beautiful turf due to its fine texture and high shoot density (Brilman 2003). For many years the only velvet bentgrass material available for breeding were vegetative clones passed from one golf course to another (Brilman 2003). Early reports show a lack of reliable seed supply as a primary reason velvet bentgrass was not used more extensively (Brilman 2003). Currently, nine varieties of this diploid species are listed (OECD 2009).

5.2 *Agrostis capillaris* L. (syn.= *Agrostis vulgaris*, *Agrostis tenuis* Sibth) – Colonial Bentgrass, Common Bent, Brown Top (Tetraploid)

Native to Europe and West Asia, sub-cosmopolitan in temperate regions. Species typical of mesotrophic to oligotrophic conditions. Predominantly cultivated for lawns and recreational turf. It is more commonly used in Europe than in the USA.

Prior to 1990, limited breeding efforts were described in Germany (one variety) and in the Netherlands (five varieties) (Ruemmele 2003). Actually 34 varieties are listed (OECD 2009). Only few varieties among them were bred for forage production. Breeding efforts have concentrated on conventional methods: germplasm collection, screening for desirable traits, polycrossing, and performance check under field conditions (Ruemmele 2003).

5.3 *Agrostis gigantea* Roth (syn. = *Agrostis alba*) – Red Top, Black Bent (Polyploid)

Native to temperate and cold areas of the northern hemisphere. Although previously widely used for forage, turf, and reclamation, it is currently of rather minor importance (Brede and Sellman 2003).

5.4 *Agrostis stolonifera* L. (syn. = *Agrostis palustris* Huds) – Creeping Bentgrass, Creeping Bent (Tetraploid)

Native to temperate and cold areas of the northern hemisphere. It is best suitable for turf due to its fine texture and adaptation to close mowing (Warnke 2003). The most popular bentgrass in the USA, widely used on golf course greens in both cool-temperate as well as warm-humid regions (Koch et al. 2007). The wide range of currently registered varieties (more than 60) reflects its perfect quality and tolerance to different soil types, salinity, and flooding. Breeding strategies used to create new creeping bentgrass varieties evolved from selection and vegetative propagation of best clones from turf areas, through mass selection to create seeded varieties, marker assisted selection, interspecific hybridization, plant transformation, and finally even genetic modification (Belanger et al. 2003, Guo et al. 2003, Warnke 2003). Creeping bentgrass is one of the first wind-pollinated, perennial, and highly outcrossing crops where a genetically modified (GM) variety was developed. However, it has been well documented that the glyphosate-tolerant trait can indeed spread to unintended or unexpected locations (Wartud et al. 2004). Therefore up till now it has not been regulated and commercialized. Modern creeping bentgrass cultivars are mostly synthetic varieties (Hurley and Murphy 1996).

5.5 *A. pratensis* L. – Meadow Foxtail

Native to Europe (excl. Mediterranean) and temperate Asia and has become naturalized in many areas outside of its native range, including North America and

Australasia. It commonly occurs as a valuable forage grass in wet meadows and is widely cultivated for pasture and hay. Although meadow foxtail possesses numerous important attributes, it received only little attention from breeders (Boe and Delaney 1996). Eleven varieties are listed (OECD 2009). Mass selection from ecotypes, increase of naturalized populations, and selection of superior parents to establish synthetic cultivars were used for the creation of new varieties (Boe and Delaney 1996). Interspecific hybridization between meadow and creeping foxtail (*Alopecurus arundinaceus* Poir.) is an important source of genetic variation useful for breeding (Boe and Delaney 1996). Seed production of meadow foxtail is difficult because of variable heading date (Figure 1) and heavy seed losses due to shattering during seed ripening. The first seed-shattering resistant cultivar 'Alko' was selected in Germany after irradiation treatment (Simon 1994).

Fig. 1 Seed production of a meadow foxtail accession in rye isolation. Developmental stage of individuals ranges from just beginning of heading to end of flowering (Photo M. Sevcikova)

An effective, yellow-leaved selection golden meadow foxtail 'Variegatus' ('Aureovariegatus,' 'Aureus') is cultivated as an ornamental color accent or in large groundcover sweeps (Darke 2004). Propagation by vegetative multiplication is required to keep the variegation trait true to type.

5.6 *A. odoratum* L. – Sweet Vernal-grass

Native to Europe, temperate Asia, and North Africa has become a sub-cosmopolitan in temperate regions. One of the earliest growing grasses in spring, resistant to harsh conditions and shadow. The only variety ('Jitka') was released in the Czech Republic (Anonymous 2005). It was bred from local ecotypes and successfully

exists on the market as a component of seed mixtures for species-rich extensive grassland. The quality of the green matter is satisfactory but the production is low.

5.7 *A. elatius* (L.) P. Beauv. Ex J. Presl and C. Presl – Tall Oatgrass, False Oat-grass

Native to parts of Europe, West Asia, and North Africa became sub-cosmopolitan in temperate regions. It is a typical species of semi-natural mesophytic hay meadows of the *Arrhenatherion* alliance from lowlands to hilly uplands and tends to expand on roadsides and banks throughout Europe and Western Asia. It has been known for a longtime in European agriculture and was introduced into the USA early in the 19th century (Wheeler and Hill 1957). It has many desirable forage traits (e.g., resistance to summer drought); however, it has only attained great importance in certain areas (Stubbendieck and Jones 1996). It is a very persistent and aggressive component of mixtures, similar to tall fescue and orchard grass (Borawska-Jarmułowicz 2004, Dembek et al. 2005). Breeding work and its cultivation as a forage grass is located mainly in Central and southern Europe. Seven varieties are listed (OECD 2009). The awned seed causes difficulties in seed production, therefore breeding has successfully focused on selecting awnless types. Such varieties were released in Poland ('Wiwena'), Germany ('Arone'), and Czech Republic ('Median'). However, when used as a component of mixtures to establish near-natural multi-species meadows, awned types are preferred (Figure 2).

A vegetatively propagated variety (*A. elatius* ssp. *bulbosum* 'Variegatum' – bulbous oatgrass) exists in many gardens due to its decorative value. Leaves have white margins with a deep bluish-green stripe running down the middle, therefore plants are excellent for the front of the perennial border in gardens.

Fig. 2 Awned seed of *A. elatius* 'Levočský' (*left*) compared to awnless seed of 'Median' (*right*) (Photo M. Sevcikova)

5.8 *Bromus catharticus* Vahl (syn. *Bromus unioloides* Kunth, *B. willdenovii* Kunth) – Rescuegrass, Rescue Grass, Prairie Bromegrass (Polyploid)

The species belongs to the section (subgenus) *Ceratochloa* and originates from Argentina and Uruguay (Stewart 1996) valued as a winter-active and palatable species on fertile soils (Rumball 1974). Short-lived perennial, occasionally cultivated for fodder and locally naturalized in south Europe (Smith 1980). In Argentina it grows spontaneously in natural and disturbed areas and is widely distributed in the Pampas (Aulicino and Arturi 2008). It is cultivated and naturalized in diverse warm temperate regions where it is a facultative autogamous hexaploid species where self-fertilization is more common than outcrossing (Aulicino and Arturi 2008). South American germplasm was utilized in developing important varieties in New Zealand and USA. Breeding in Europe is mainly in France and Italy. Currently 21 varieties are listed (OECD 2009).

5.9 *B. inermis* Leyss. – Smooth Bromegrass, Hungarian Brome

Native to central and northern Europe as well as temperate Asia. Introduced to USA from Hungary, northern Germany, and Russia (Vogel et al. 1996). Adapted to dry climates and very resistant to drought. Despite of its European origin, almost one-third of all registered varieties were bred in the USA and Canada. From the end of the 19th century till the 1930s smooth bromegrass did not gain wide acceptance in North America (Casler et al. 2000). However, after the heavy droughts during the 1930 s, it received more attention (Vogel et al. 1996). In the USA, two types of smooth brome differing in appearance and growth habit are recognized; they are known as northern (late-producing) and southern (early-producing) strains (Wheeler and Hill 1957) and several cultivars of both types have been developed (USDA 2009). The first cultivars were released after selection among existing ecotypes. Inbreeding work on smooth brome grass yielded no varieties, mostly due to problems with controlling pollination in seed multiplication fields. During further development of varieties, half-sib progeny testing was applied (Vogel et al. 1996). Despite of huge efforts made, forage yields of smooth bromegrass have been increased only about 5–10% in over 50 years of breeding (Vogel et al. 1996, Casler et al. 2000). Presently about 34 varieties are registered (OECD 2009).

An ornamental, variegated cultivar ‘Skiners Gold’ with leaves mostly green-margined and broad, light yellow variegation is cultivated and propagated vegetatively for gardening purposes (Darke 2004).

5.10 *Bromus marginatus* Nees ex Steud. – Mountain Brome

Short-lived perennial bunchgrass native to mountain and subalpine zones in Cordillera and western part of Great Plains. It belongs to the section (subgenus)

Ceratochloa. In the USA, four distinct types are distinguished – short early, tall late, intermediate, and hairy drought tolerant. In the between-the-wars period, mountain brome was introduced to Europe and cultivated in short-term forage grass mixtures in Czechoslovakia (southern Bohemia). In 1998, the variety 'Tacit' was registered in the Czech Republic. An intermediate variety 'Bromar' was developed in the USA (Wheeler and Hill 1957), which was later replaced by 'Garnet' with improved persistency (USDA 2009).

5.11 *Bromus sitchensis* Trin. – Alaska Brome (Upland Brome in NZ)

Smooth, perennial up to 180 cm tall (Hitchcock 1971). Similar in appearance and potential use to *B. inermis.* Alaska brome is native to West Coast of North America (from Alaska to California). This perennial species belongs to the section (subgenus) *Ceratochloa.* Alaska brome can be used as a companion with alfalfa. It is fast in establishment and regrowth. Four varieties are listed.

5.12 *Bromus stamineus* E. Desv. – Southern Brome, Roadside Brome (Grazing Brome in NZ)

Native to Southern America (Argentina, Chile, Peru), naturalized in New Zealand and the Western part of the USA. This perennial species belongs to the section *Ceratochloa.* Four varieties are listed (OECD 2009).

5.13 *C. cristatus* L. – Crested Dog's Tail Grass, Crested Dogtail

Native to the Europe and the Caucasus. Caespitose, with loose tufts consisting chiefly of leafy shoots with a short rootstock. It is a common grassland species, found predominantly in pastures, but also in old meadows. 'Rožnovská,' probably the first variety, was registered in the Czech Republic in 1940 and was recommended for pastures and playgrounds. It is still available and in Switzerland it was quite recently found as agronomically recommendable for pastures in higher altitudes together with the varieties 'Tercie' (='ČD-1,' Czech Republic), 'Cristal' (Austria), and 'Cresta' (Switzerland) (Suter et al. 2004). The Olympic Stadium in Munich in 1972 was seeded with a grass mixture with 15% share of 'Credo Sceempter,' the Dutch variety of crested dog's tail (Gollwitzer 1972). Currently four varieties are available (OECD 2009).

5.14 *D. cespitosa* (L.) P. Beauv. – Tufted Hairgrass, Tufted Hair-Grass

Native to temperate and cold areas of the northern hemisphere and to the mountain areas of tropical Africa. Very complex species, with many variants which differ to a greater or lesser degree in ecology, cytology, and morphology (Clarke 1980, Davy 1980, Żurek 2000).

First information concerning breeding of tufted hairgrass is available from the late 1960s and refers to decorative and bunch-type varieties (Zeller 1969). Breeding for turf and re-vegetation purposes in this species has been initiated only recently (Brilman and Watkins 2003). However, seven cultivars are currently listed (OECD 2009). Tufted hairgrass is of great potential for use as turfgrass with low nitrogen and light requirements, for erosion control, reclamation, and heavy wear sites (Brilman and Watkins 2003). All current breeding programs use wild germplasm. Breeding objectives are billbug and rust resistance, and increased heat tolerance (Prończuk et al. 1996, Prończuk and Czembor 1998, Brilman and Watkins 2003).

Intraspecific variability in inflorescence color led to the development of vegetatively propagated ornamental cultivars valued for the cloud-like quality of their inflorescences in various shades of green to gold. A cream-white variegated selection 'Northern Lights' found in Nebraska has not been observed to flower (Darke 2004).

5.15 *H. lanatus* L. – Common Velvetgrass, Yorkshire-Fog

Native to Europe, West and East Asia, North Africa, and North America has become sub-cosmopolitan especially in temperate regions. Yorkshire fog is a tufted, perennial grass, commonly found in rough grassland, pastures, wasteland, and open woods, but of inferior forage quality. Only two varieties are listed (OECD 2009). Non-European varieties were bred as a good yielding forage crop on soils of lower fertility (USDA 2009). The Czech variety 'Hola' was selected from indigenous ecotypes and was registered for non-agricultural purposes as a pioneer grass for poor soils and a component for extensive wet grassland mixtures. The diploid variety 'Massey Basyn' was colchicine treated to create tetraploid germplasm 'G44' in New Zealand (Rumball and Miller 2005).

5.16 *K. macrantha* (Leder.) Schultes – Prairie Junegrass, Junegrass, Crested Hairgrass

Widely distributed in Europe, from North Scotland to Lithuania, casual in Fennoscandia as well as in temperate regions of the New World (Hitchcock 1971, Humphries 1980). This is a complex species with large natural variation as a result of polyploidy and adaptation to a wide range of ecological conditions (Humphries

1980, Dixon 2000). Therefore all three currently available varieties were selected from ecotypes (USDA 2009, Soovali and Bender 2006) with potential use as cool-season turfgrass.

5.17 *P. arundinacea* L. – Reed Canarygrass, Reed Canary-Grass

Native to northern temperate regions and widely distributed throughout Europe, Asia, America, and Africa. However, it is disputed whether it is native to North America or was introduced from Europe. North American populations may be mixtures of native populations and European cultivars (Sahramaa 2004). Breeding programs of reed canary grass have aimed at increased forage and seed yield, improved forage palatability, and to decrease the concentration of alkaloids (tryptamine and carboline) harmful for cattle and sheep (Gyulai et al. 2003, Sahramaa 2004). In addition to selections from natural germplasm, synthetics based on pre-selected clones and also somaclones have become new sources of variation in reed canarygrass breeding (Gyulai et al. 2003). Populations of tissue culture-derived somaclones were found to be useful for generating new reed canarygrass breeding material. Interspecific hybridization has also been used in reed canary grass breeding. The possibilities for the transfer of the intact rachilla trait of *Phalaris aquatica* to reed canary grass and recombination of the palatability of *P. aquatica* and winter hardiness of *P. arundinacea* have been both of interest by breeders (Sahramaa 2004). To make the species suitable for forage, high alkaloid content has been decreased through breeding (Carlson et al. 1996). New breeding aims were recently specified for industrial use (energy and fiber) of reed canary grass (Wrobel et al. 2009). The first non-fodder cultivar 'Bamse' has been recently released in Finland (Sahramaa 2004). Presently 13 varieties are listed (OECD 2009).

A few decorative forms (in fact botanical varieties) of reed canary grass, propagated only by vegetative multiplication of old plants exist in many gardens. Varieties like 'Picta' (*P. arundinacea* var. *picta* – ribbon grass), 'Luteopicta' (*P. arundinacea* var. *luteo picta* – golden ribbon grass) 'Feesey,' 'Tricolor,' and 'Woods Dwarf' of white-green or yellow-green striped leave blades are good candidates for ground cover or garden borders. These varieties quickly colonize and serve as a dense, weed-free ground cover in areas where aggressiveness is not a concern (Darke 2004).

5.18 *P. distans* (L) Parl. – Weeping Alkaligrass, Reflexed Saltgrass, Alkaligrass)

Native to temperate Eurasia and northern Africa and widely naturalized in temperate regions. A species for very specific purposes with only a few varieties. Weeping alkaligrass is one of the most salt-tolerant grasses available (Hughes et al. 1975). Considering its turf value (resistance to close mowing, slow regrowth)

associated with tolerance to drought, shade, and cold, it is a superior turf component on shorelines, roadsides, airports, wetlands golf courses, and naturalized areas where problems with salt spray, salt water intrusion, or chemicals from melting ice occur. Alkaligrass is useful in mixtures where salt is a problem but it is crowded out by other grasses on fertile and well balanced soils (Brede 2000).

5.19 *T. flavescens* (L.) P. Beauv. – Golden Oatgrass

Golden oatgrass is native to Europe, west Asia, and north Africa, and naturalized in North America and southern Latin America. It is a valuable component of higher altitude meadows and has been bred and cultivated as a forage grass, especially in central European countries. Some of them have been used in mixtures for permanent grassland for decades. Yellow oatgrass belongs to the calcinogenic plants which induce calcinosis in cattle, sheep, goats, pigs, horses, etc., when they graze the swards containing more than 20% yellow oatgrass for a longer period (Mello 2003). The prevention of calcinosis is important in the Alpine regions (Franz et al. 2007). Eight varieties are listed (OECD 2009).

6 Seed Production

All grass species described in this chapter belong to the group of small- to medium-seeded grasses except brome grasses and tall oatgrass with bigger seeds. Their seed production forms only a small part of grass seed agribusiness. In the EU, total grass seed production occupied about 250.000 ha producing approximately 257.000 tons in 2006 and in these figures only bentgrasses and tall oat grass were included. These two representatives of minor grasses genera were grown on only 0.30% of the total acreage with an annual production of 338 t (0.13% of the total production). The major part belongs to tall oat grass and bentgrasses for non-agricultural or amenity purposes. According to the world statistic of the International Seed Federation, the seed production of bentgrasses was 5.567 t at 2006 in USA (ISF 2006). In brome grasses, Argentina is the largest producer (3.921 t), followed by the USA (1.167 t); the European seed production is represented by France with 149 t; other minor grasses are not included in the statistics.

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Alfalfa

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1 Introduction

Cultivated alfalfa (*Medicago sativa* L., 2n = 4x = 32), often called "Queen of the forages" (Barnes et al. 1988), is a tetraploid perennial, open pollinated legume with polysomic inheritance. Native to the Middle East, alfalfa belongs to the *M. sativa–falcata* complex, where interfertile diploid and tetraploid forms coexist (Quiros and Bauchan, 1988). In modern agricultural production systems, alfalfa can be harvested for up to 4–5 years before the stand deteriorates, although rotation to other crops after 2–3 years is common. In northern areas, seeding can be performed in spring or early autumn; autumn seedings are most common in southern production regions. Recommended seeding rates are very variable across locations and soil types, typically from 10 to 25 kg ha^{-1} in pure stand.

In a large part of the Americas and Europe, alfalfa is the most important forage legume, and is grown for hay, dehydrated forage, pellets, silage, and occasionally, grazing. Alfalfa residues increase soil organic matter, and its root system mobilizes nutrients deep within the soil profile and improves soil structure, permeability to water, and water retention capacity. Furthermore, alfalfa cultivation only requires low inputs of herbicides and pesticides and no N fertilizers due to N-fixation by the symbiont *Sinorhizobium meliloti*. These characteristics are favorable with regard to national and international policies and public concern about the environmental impact of agricultural activities. Alfalfa hay fields are also highly favorable to biodiversity enrichment. Finally, the high forage protein content of alfalfa meets the needs of the feed market, particularly since concentrates of animal origin were banned in the European Union (Veronesi et al. 2006).

USA, eastern Europe, and Argentina contribute about 70% of alfalfa forage production area, while France, Spain, Italy, Canada, China, and Australia contribute about 20%. Estimates from the second part of the 1980s reported that alfalfa was

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grown on more than 30 million ha worldwide (Michaud et al. 1988) and the estimates remained unchanged at the beginning of the 21st century despite a sharp decrease in the eastern European countries as political changes in the last decade of the 20th century strongly affected agricultural systems. These decreases were counterbalanced by increases of the production area in Australia and China, which could mark the beginning of a significant shift in the main regions where the crop will be grown in the future (Bouton 2001).

2 Origin and Systematics

Alfalfa belongs to the very large *Medicago* genus, which is predominantly centered around the Mediterranean basin. This genus is related to *Trigonella* and *Melilotus*, which have very similar leaf features. The *Medicago* genus comprises species widely varying in morphological characteristics. About two-third of the species are annuals (Lesins and Lesins 1979). The taxa constituting the *M. sativa–falcata* complex belong to the section *Falcago*, subsection *Falcatae* within the genus *Medicago*, which includes diploid and tetraploid forms.

The *M. sativa–falcata* complex includes a series of subspecies at both ploidy levels (Figure 1). All members of the complex share the same karyotype (Gillies 1972). Both diploid (2n = 2x = 16) and tetraploid (2n = 4x = 32) forms exist within the complex.

M. sativa subsp. *falcata* has straight to sickle-shaped pods and yellow flavonoids and carotenoids in the petals. The diploid and tetraploid forms are distributed over a wide geographical range, from eastern France (Malzeville in Lorraine) in the west to Siberia and inner Mongolia in the east and from the Black Sea coast in the south to Leningrad in the north. The taxon is well adapted to cold regions, where tetraploid forms occur at higher frequencies than the diploids. Subsp. *falcata* is highly variable in morphological and biochemical traits, which may explain the diversity of names and species or subspecies rank that diploid forms have been given by various authors, such as *borealis, romanica, altissima,* or *glandulosa*.

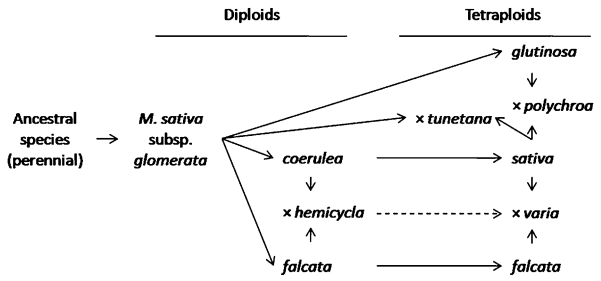

Fig. 1 Taxonomic relationships among subspecies of the *M. sativa* complex. All taxa listed are subspecies of *M. sativa*. (After Quiros and Bauchan 1988)

Diploid and tetraploid forms of purple flowered *M. sativa* are named subsp. *caerulea* (also denoted as *coerulea*) and subsp. *sativa*, respectively. Both show coiled pods and anthocyanins in the petals which lead to violet or lavender flowers. The range of distribution covers a wide territory including the Mediterranean area, the Near and Middle East, the Caucasus, and Middle, Central, South and eastern Asia. The highest variability of the species is concentrated at the foothills and mountain valleys of Armenia, Iran, Afghanistan, and Central Asia (Ivanov 1977).

The hybrids between *sativa* and *falcata* subspecies are easily produced and are fully viable and fertile. They show coiled pods and variegated flowers. According to Lesins and Lesins (1979), the hybridization of subsp. *sativa* and subsp. *falcata* might have contributed to the cultivation of alfalfa throughout much of the temperate zone by generating a huge range of genetic variability for all adaptive traits, offering a potential for adaptation to most climates.

Medicago glomerata, a diploid subspecies, is characterized by bright yellow flowers, coiled pods, and glandular hairs on stems. It is predominantly found in southern Europe, the Alps, and North Africa. The tetraploid form of subsp. *glomerata* is subsp. *glutinosa*, which has bright yellow or cream corolla color at bud stage changing to full yellow several hours after opening, coiled pods, and glandular hairs on stems. It is adapted to moist, subalpine environments. According to Lesins and Lesins (1979), this subspecies may originate from crosses between diploid *M. sativa* subsp. *falcata* and *M. sativa* subsp. *glomerata* another diploid species from the *M. sativa* complex.

Using morphological and isozyme data, Quiros and Morgan (1981) and Quiros and Bauchan (1988) proposed an evolutionary pathway of the *M. sativa–falcata* complex and their closely related species (Figure 1).

Molecular marker analysis of both wild and cultivated populations showed that the domesticated pool contained on average 31% less diversity than the wild pool, but with high heterogeneity among loci. Simulations of the domestication process were consistent with a demographic bottleneck during domestication (Muller et al. 2005).

Michaud et al. (1988) proposed an overview of the geographical movement of alfalfa. Alfalfa likely had two centers of domestication, in Transcaucasia and Minor Asia and in Central Asia, from which it spread worldwide. The name "alfalfa" derives from the ancient Persian word "aspast" meaning horse fodder. Alfalfa spread to Gansu province in Northwest China some 3000 years ago (Gen et al. 1995) and was reported in Turkey by 1300 BC and Babylonia by 700 BC. The Romans introduced it into Italy in the second century BC. Columella planted alfalfa in Andalusia in southern Spain in the first century AD. It may have been introduced to France at the same period of time but did not expand. De Serres (1600) reported alfalfa as a very useful crop for improving soils, mentioning for the first time that the name "luzerne" was used for the crop in Provence-Languedoc. "Luzerne", transliterated to "lucerne" in English, is thought to derive from "luzerno" meaning "shiny" in patois provençal and referring to the shiny appearance of alfalfa seed. However, alfalfa or lucerne was very little used until the mid-1700s when perennial forage legumes

(alfalfa, sainfoin, and red clover) became widely used to produce hay for military horse herds and to improve soil fertility (Gilbert 1789).

The discovery of the Americas and their colonization by Portuguese and Spaniards in the 16th century led to the introduction of alfalfa into Mexico and Peru. From these initial introductions, alfalfa first moved to all countries in South America, reaching the last country, Uruguay, in 1775 (Klinkowski 1933). Alfalfa was introduced into Texas, Arizona, New Mexico, and California by missionaries in the early 1800s. Material from South America was also introduced in the mid-1800s and was very well adapted (Hendry 1923). Between 1858 and 1910, winter-hardy germplasm sources were brought from Europe and Russia into the upper midwestern USA and eastern Canada. In addition, two intermediate winter-hardy materials were introduced, one from a broad area in the Near East and the other from France in 1947. Non winter-hardy populations were introduced from Peru (1899), India (1913 and 1956), and Africa (1924). Ultimately, nine historical germplasm introductions into the United States have been recognized, including *M. sativa* subsp. *falcata*, *M. sativa* subsp. *varia*, Ladak, Turkistan, Flemish, Chilean (Spanish), Peruvian, Indian, and African (Barnes et al. 1977). More recent introductions from the Arabian Peninsula have expanded the USA germplasm further (Smith et al. 1995). Subsequent reintroductions from around the world have continued until the present day, considerably mixing the cultivated germplasm currently in breeding pools.

3 Genetic Resources and Utilization

The genetic resources of *M. sativa* subsp. *sativa* are predominantly landraces and traditionally cultivated populations. For subsp. *falcata* and the related diploid subspecies, wild populations exist and may be collected and exploited. Most landraces and cultivated populations in the temperate zones were collected, analyzed, and exploited for breeding in the early steps of alfalfa breeding. They constituted the original pool from which numerous varieties were selected.

The basic germplasm used for alfalfa breeding in the USA correspond to phases of introduction of genetic diversity into North America as described above (Barnes et al. 1977, 1988). These sources show considerable variation in agronomic value and physiological traits, especially winter hardiness and fall dormancy, with subsequent effects on biomass production and on genotype $\times$ environment interaction for production and adaptation. Large differences exist among germplasm sources at a similar phenological stage for nutritive value; for instance, Indian and Flemish germplasm had a higher crude protein content than Turkistan and Peruvian at a late bloom stage (Lenssen et al. 1990). Germplasms also exhibited different rates of decline in forage quality.

Landrace populations traditionally cultivated in local regions throughout the world are potential sources for key adaptation traits, for diversifying breeding pools, and for investigating possible heterosis. For example, germplasm collected in the Arabic peninsula and Yemen offer high genetic diversity for growth in dry

conditions and marked non-dormancy (Smith et al. 1995). In Spain, a group of wild populations called Mielgas represent a very peculiar germplasm that has a very prostrate growth and that may produce rhizomes. This germplasm could be a valuable source for breeding for adaptation to grazing and to drought-prone environments (Prosperi et al. 2006). Mitochondrial diversity showed that the Mielgas constitute an endemic wild pool which was progressively introgressed by cultivated alfalfa (Muller et al. 2003). Their divergence for nuclear sequence polymorphism with the cultivated alfalfa was small (Muller et al. 2005).

Germplasm from Egyptian oases, especially in the Siwa region, represent populations traditionally cultivated by farmers in small irrigated fields with little seed exchange among farmers. On the basis of 76 agronomic and morphological characters, large differences were observed between the oasis populations and varieties registered in Egypt and mainly cultivated in the Nile valley. The Siwa landraces were characteristically taller at different measurement dates. An analysis of SSR markers showed that the Siwa landraces clustered together but were distant from the Egyptian cultivated material and even more from the Italian varieties used as external checks. The variation among populations from the oasis, though significant, was smaller than expected under the hypothesis of limited seed exchange. This would suggest that seed exchange among farmers may occur or that pollen transport among fields is intense enough to homogenize the material (Carelli et al. 2009). This material from the oasis could be extremely valuable for its tolerance to high temperatures and its summer growth.

Alfalfa is of increasingly interest for Chinese farmers because of its potential for high levels of protein production in dry conditions. Currently, research to investigate the diversity available among traditionally cultivated populations of various Chinese regions and especially in Gansu province in Northwest China is underway (Wei, 2004; Hu et al. 2000; Wang et al. unpublished data). Recent synthetic cultivars appear to be similar to foreign germplasm and distinct from the native ecotypes, suggesting the use of cultivar introductions in breeding. This underlines the need to pursue collection and evaluation of local populations for future use.

In Iran, alfalfa is important, especially in the west and northwest part of the country. Cultivated alfalfa is based only upon traditional populations. An analysis of general combining ability for dry matter yield among a set of 29 populations suggested wide genetic variation is present in cultivated germplasm, which represents a valuable source for breeding programs (Monirifar, unpublished data). The genetic distance between these populations and material available in gene banks such as the USDA-National Plant Germplasm System (www.ars.grin.gov/npgs/) should be investigated.

4 Varietal Groups

The vast majority of alfalfa cultivars belongs to the subspecies *sativa* and × *varia*. This latter group represents a range of introgression of the *falcata* genome into the *sativa* genome. These cultivars are characterized by the presence of a certain

Fig. 2 Alfalfa plants with *purple* and *yellow* flowers and their hybrid progeny (*center*) showing variegated flower color (photo Xuehui Li)

percentage of plants with variegated flowers (Figure 2). A few cultivars belonging to subsp. *falcata* have been developed, such as "Anik", a diploid variety.

Cultivars are characterized by a wide range of agronomic and physiological traits. In the USA, the North American Alfalfa Improvement Conference (NAAIC) has developed a range of standardized tests for a proper assessment and characterization of varieties (http://www.naaic.org). In Europe, the characterization is made during the process of registration, especially with the DUS tests and in VCU trials.

Fall dormancy is a crucial trait for characterization as it partially determines the possible area of cultivation. It influences the regrowth rate after each harvest and is generally associated with winter survival and frost resistance (Smith 1961). However, the relationship between strong fall dormancy and high winter survival is not very close and winter hardiness has to be assessed separately with adequate tests.

A standardized test was developed to test fall dormancy based on a range of check cultivars (Table 1). After the 1998 revision, 11 dormancy classes are classified in the North American system (Teuber et al. 1998). The test is based on the plant height of regrowth after a late clipping in autumn. It is run on transplanted plants (Viands and Teuber 1985). In order to run similar tests under other soil and climate conditions

Table 1 Fall dormancy classes and designated check cultivars

Fall dormancy class	UPOV listed checks
1	Maverick
2	Vernal
3	Boja, Ranger
4	Legend, Mercedes, Cutter
5	Archer, Dupuits
6	ABI700, Dorine
7	Dona Ana, Sutter, Oro
8	Maricopa, Carmen, 5715
9	CUF101, Carmen, 5929
10	UC-1887
11	UC-1465

(Montegano et al. 2002), more checks were added and are now listed in the UPOV guidelines (Table 1).

In Europe, alfalfa varieties (www.OECD.org) are often classified into two main groups, i.e., the Flemish type mainly used in northern regions and the Provence or Mediterranean type to be used in the southern regions under a Mediterranean climate. These two groups are clearly separated based on morphological traits, but molecular markers did not differentiate among them (Herrmann et al. 2009). The cultivar "Luzelle", adapted to grazing and showing a prostrate growth habit due to a high proportion of *falcata* plants in its parentage, was separated from both groups based on both morphological traits and molecular markers. The variation for both morphological traits and molecular markers within groups was large enough to differentiate between pairs of cultivars. Because of the autotetraploid structure of the alfalfa genome and cross pollination, alfalfa cultivars typically exhibit broad genetic variation.

As much as 99% of the variation for molecular markers was detected within cultivars (Herrmann et al. 2009), with a similar pattern found by Flajoulot et al. (2005) on a set of cultivars from a single breeder. Although the cultivars were distinct based upon morphological traits measured for the DUS test, on average, they shared 99% of their neutral genetic background revealed by SSR markers. This may be explained by the fact that the selection focuses on a few traits controlled by a finite number of genes which are not distributed over the whole genome.

A broad genetic variation within cultivars may be extremely valuable when a new trait needs to be selected, such as a new disease resistance. Indeed, in such a situation, breeding may be carried out within the existing cultivars and breeding pools where genetic variation for the new trait may exist. For instance, resistance to stem nematode in the European Flemish type cultivars, which had initially a low percentage of resistance, was increased as rare resistant genotypes were defined and used in a recurrent selection process. Large variation may improve the adaptation of cultivars to a wide range of environments. However, it may slow down genetic progress by slowing the concentration of desirable alleles and limiting the purging of deleterious alleles.

5 Major Breeding Achievements

Yield still remains the most important breeding target for alfalfa and therefore it deserves a particular attention. The genetic increases in alfalfa yields have been small compared with those achieved in most grain crops (Hill et al. 1988). Hill and Kalton (1976) estimated that in the USA total genetic yield improvement between 1956 and 1974 was about 3%. In Europe, in the last decades it is evident that alfalfa dry matter yield increase in comparison with old cultivars and local ecotypes has been very limited, being not more than 5% (Lloveras et al. 1998; Kertikova and Scotti, 1999; Babinec et al. 2003; Delgado et al. 2003; C. Huyghe and F. Veronesi, unpublished data) (Figure 3).

Fig. 3 A modern variety of alfalfa at flowering (Photo R. Torricelli)

An annual rate of yield increase of 0.18% was found in Wisconsin, USA for cultivars representing different eras of breeding from 1898 to 1985 (Holland and Bingham 1994). The authors suggested that favorable alleles have been accumulated in modern alfalfa cultivars but that this mostly occurred between 1900 and 1950, while increased heterozygosity or exploitation of non-additive genetic effects may account for much of the improvement in cultivar yield potential that occurred between 1950 and the present time. A more recent experiment conducted in the midwestern USA suggested that in the absence of significant disease pressure, yields over the past 60 years have not changed appreciably, but where disease pressure was present, new cultivars show a distinct yield advantage (Lamb et al. 2006).

In the opinion of Veronesi et al. (2006), the slow genetic gains are largely due to: (i) the very good adaptation of local populations; (ii) the wide genetic basis of the cultivars; (iii) the small number of research teams devoted to applied alfalfa plant breeding; and (iv) the inherent difficulties connected to the tetrasomic inheritance of the species. Some other reasons for the limited gain for yield, at least in the USA, are that yield per se is often not explicitly selected and that selection methodologies ideally suited to improving complex quantitative traits, such as progeny testing, are rarely used (Brummer 2005; Casler and Brummer 2008).

The presence of important environmental influences on varietal performance makes the investigation of genotype × location interaction of special interest for alfalfa (Annichiarico and Piano 2005). The identification of morphological traits associated with general and specific adaptation patterns could possibly be coupled, in the long run, with molecular markers to improve the understanding of genotype × location interaction effects as well as assist plant breeding efforts aimed at definite adaptation targets (Annichiarico 1999).

In addition to yield, feeding value is an important trait for alfalfa, which is characterized by high protein content but moderate energy value. Near infrared spectroscopy (NIRS) calibrated by wet chemistry laboratory measurements of enzymatic digestibility, neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid

detergent lignin (ADL), make assessment of feeding value sufficiently high throughput for breeding purposes (Andueza et al. 2001; Odoardi et al. 2001). Large genetic variation was observed among cultivars and among individual plants (Julier et al. 2000), and the inheritance of feeding value proved to be mainly additive (Guines et al. 2002a).

Alfalfa contains proteins which are rapidly degraded in the rumen, inducing a poor dietary efficiency, risk of bloat, and nitrogen loss detrimental to the environment. Investigation of variation in ruminal protein degradability in alfalfa suggests that the range of genetic variation within cultivated alfalfa for in situ crude protein (CP) degradation appears to be narrow (Julier et al. 2003a). Similar results have been obtained in a practical breeding program by Torricelli et al. (2001), who did not find a large variability for CP among alfalfa plants. Even though no negative correlation between DMY and CP was observed, from a practical point of view, too many single plants had to be analyzed to apply a sufficient selection differential for CP within alfalfa populations. Thus, starting a specific breeding program does not appear as worthwhile as identifying variability for CP among alfalfa populations. Useful genetic variation for quality components identified in germplasm has been used to develop grazing type alfalfa cultivars (Pecetti et al. 2001).

On the basis of the results discussed above, new selection criteria have been added to alfalfa breeding programs to create cultivars with higher fiber digestibility and with high agronomic performances. The target is not a simple one, due to the negative genetic correlation between digestibility and forage yield (Julier and Huyghe 1998). Molecular mapping is underway in France and in the USA with the objective of identifying genetic markers associated with growth and digestibility traits to obtain a better understanding of the relationships among these traits and to produce genetic marker tool kits useful for alfalfa breeders. Choosing cultivars for dehydration focuses mainly on yield and disease and pest resistances rather than quality, because cultivar differences in dehydrated product quality are small relative to variability in agronomic technique, harvest frequency, and weed control (Corsi et al. 2001). As a consequence, breeding approaches for quality are unlikely to be sustained by private breeders in the absence of a clear economic return in terms of higher market prices.

Another important trait is grazing tolerance. Traditional cultivars generally exhibit lower persistence under grazing management relative to mowing. In the last two decades, alfalfa grazing has aroused new interest due to the need for extensive livestock systems, either to reduce the environmental risks related with intensive animal husbandry or to decrease the costs of production as a way to maintain competitiveness of livestock enterprises. On the basis of these considerations, several research teams started selection programs for grazing tolerant alfalfa (Delgado Enguita 1989; Smith and Bouton 1993; Charrier et al. 1993; Piano et al. 1996; Smith et al. 2000). In the USA, a standard test to assess the grazing tolerance of alfalfa cultivars has been developed (Bouton and Smith 1998). Some cultivars with a better survival under grazing were released, including “Coussouls” and “Luzelle” in France and “Alfagraze” in the USA (Bouton et al. 1991). “Alfagraze” was the first of a series of dual purpose alfalfa cultivars released in the USA which had acceptable

hay yield as well as grazing tolerance. These cultivars were developed by selecting surviving plants from grazing trials. By selecting from within high yielding cultivars, the productivity of the resulting cultivars was much higher than the typical low yield of most grazing tolerant cultivars. Research is still in progress (Pecetti and Piano 2005) and alfalfa cultivars from European germplasm specifically adapted to grazing will enter the seed market in Europe in the near future.

Winter survival is a trait of particular importance in the northern regions of alfalfa's cultivation range (Castonguay et al. 2006). Winter survival is related to, but not the same as, fall dormancy (Brummer et al. 2000), and the NAAIC has developed a standard test to characterize winter survival separately from fall dormancy (McCaslin et al. 2003). Breeders have made significant improvements in winter hardiness. Three cycles of recurrent selection in nondormant germplasm resulted in populations with excellent winter survival in the upper midwestern USA. (Weishaar et al. 2005). A particularly noteworthy selection methodology has been developed in Canada in which controlled freezing in the laboratory has resulted in substantial gains in winter survival (Castonguay et al. 2009). Developing tightly controlled laboratory tests analogous to this freezing test for other complex abiotic stresses, such as drought or aluminum tolerance, should have a high priority for alfalfa breeders in forthcoming years.

Other selection targets are improved seed yield (Svirskis 1997; Huyghe et al. 1999), resistance to pea aphid (Bournoville et al. 2001), persistence under frequent cutting regimes (Nagy 2003), increased root size (Saindon et al. 1991; Chloupek and Skácel, 1999), salt tolerance (Hefny and Doliski, 1999), and tolerance to soil acidity and aluminum toxicity (Hauptvogel, 1999).

6 Specific Goals in Current Breeding

6.1 Chemical Composition and Feeding Value

Feeding value continues to be a major concern for alfalfa breeders. Alfalfa is a major source of protein for ruminants, but when included in diets of highly productive animals, alfalfa provides insufficient energy to adequately use the nitrogen because of its relatively low digestibility. Moreover, during the process of growth and biomass accumulation, both protein content and digestibility decline. The decreasing protein content is a dilution effect related with the decreasing leaf to stem ratio; the leaves have a stable protein content and their protein level is much higher than the protein content of stems. The decline of digestibility is the consequence of two processes: (i) the reduction of a highly digestible component (leaves) because of an increase of a less digestible component (stems) and (ii) the decreasing average digestibility of the stem component, with more cell walls (NDF) and lignin. The proportion of NDF also influences voluntary intake. For a similar level of energy, animal intake of alfalfa needs to be higher than intake of forage grasses, consequently resulting in excretion of excess nitrogen.

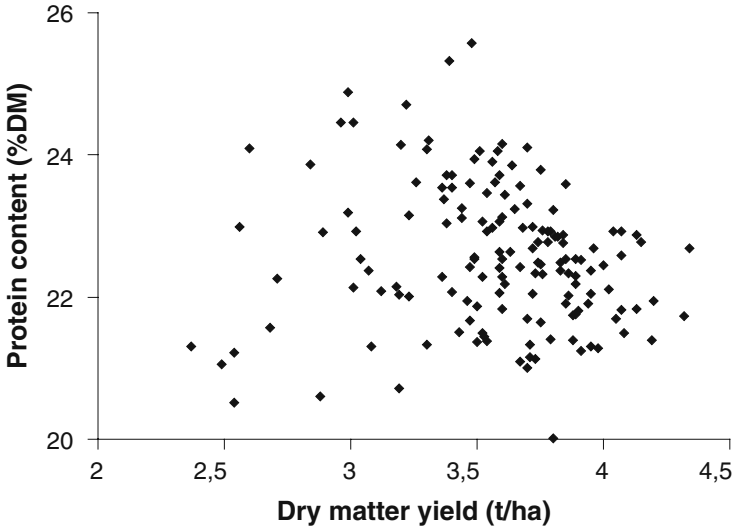

Fig. 4 Relationship between dry matter yield and protein content of 150 alfalfa cultivars at the first harvest (source: GEVES, France)

Protein content has a negative genetic correlation with biomass yield (Figure 4). The figure shows that variation in protein content exists among high yielding cultivars, suggesting that genetic gain may be made for both yield and protein content.

At the genotypic level, a negative relationship exists between dry matter yield and digestibility. However, this relationship is not too severe and breeding for both dry matter yield and improved digestibility is possible. Interestingly, under French conditions where lodging resistance is an important feature, no relationship between yield and digestibility was detected. It is thus possible to have cultivars with a high biomass production and a high digestibility. The combination of yield and digestibility requires modifying the proportion of cell walls and their distribution within the stems (Guines et al. 2002b) and improving cell wall digestibility.

Jung and Lamb (2006) carried out a divergent selection for *in vitro* cell wall digestibility measured either at 16 or 96 h. Positive selection for cell wall digestibility at 16 h led to a reduction of cell wall content. Selection for increased digestibility at both 16 and 96 h resulted in a reduction of the lignin content and an increase in the pectin content of plants. Divergent selection on the cell wall fractions showed that reducing ADF (acid detergent fiber, roughly comprised of cellulose and lignin) and lignin content increased dry matter digestibility (Tecle et al. 2008). However, these two studies did not report the consequences of selection for improved nutritive value on the dry matter yield in swards. Finally, selection for higher pectin content resulted in a reduction of cell wall content (NDF and ADF) and better dry matter digestibility (Tecle et al. 2006).

Crude protein content and/or dry matter digestibility are now taken into account for cultivar registration in some countries. In France, both protein content and ADF content are measured and contribute to the registration index. Figure 5 shows the values of checks and candidate cultivars observed over the last three cycles of cultivar analysis. It clearly shows that it is possible to make progress on components of feeding value without negative effect on the global agronomic value, where dry matter yield is very important.

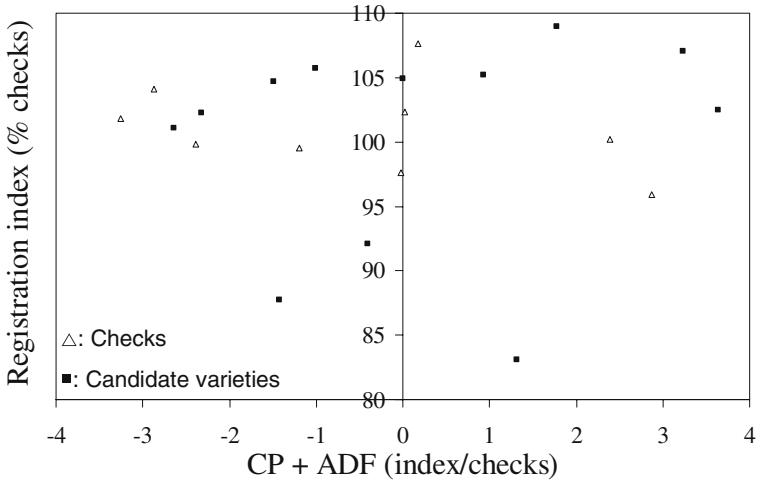

Fig. 5 Relationship between the improvement of chemical composition and the final registration index for candidate cultivars and checks during cultivar testing (source: GEVES, France). On the *x*-axis, the difference between the quality indices of each variety, based on crude protein (CP) and ADF content, and the mean quality index of checks is shown

6.2 Disease and Pest Resistance

Resistance to diseases and pests has been a major focus of breeding efforts over the past 50 years, resulting in substantial genetic progress for the main biotic constraints to alfalfa cultivation in most regions. Standardized tests have been developed for many of the most significant biotic stresses (see http://naaic.org/stdtests/index.html). The achievements may be illustrated in improvements of resistance to pests, fungi, and bacteria.

Pests. In North America and Australia, most breeding effort has been devoted to resistance to various aphids. In Europe, stem nematode (*Ditylenchus dipsaci* (Kuhn) Filipjev) has been a major biotic stress over the last three decades. Screening methods adapted to breeding, enabling the evaluation of large numbers of individuals using standardized tests, have been implemented for the various pests.

Available germplasm was screened for stem nematode resistance (Julier et al. 1996) and various sources were identified. However, the within-pool genetic

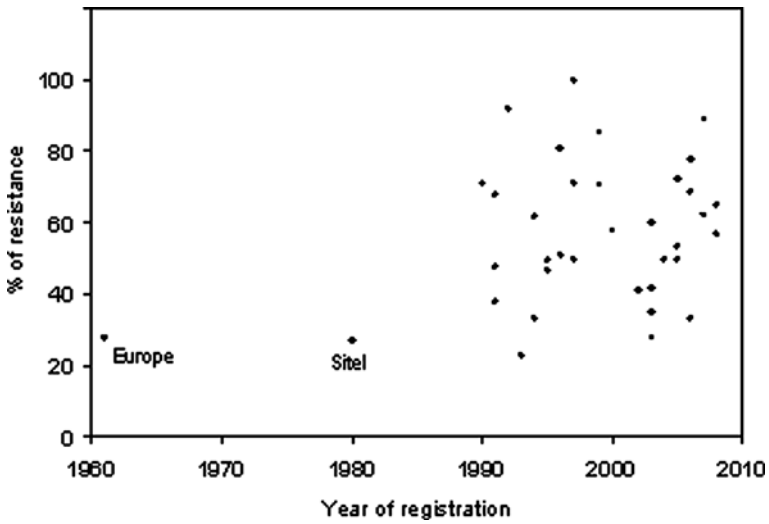

Fig. 6 Percentage of resistance to stem nematode of the cultivars registered on the French national catalogue as a function of the year of registration

variation was sufficient to improve stem nematode resistance without significant introgression of exotic germplasm. Major genetic gains have been made in France over the last three decades (Figure 6). In comparison to two old check cultivars, "Europe" and "Sitel", new cultivars have higher levels of resistance.

Fungi. Alfalfa may be damaged by a wide range of fungi which will negatively influence biomass production, forage quality, and sward persistence. As a consequence, a broad range of pathogens has been taken into account in breeding programs: verticillium wilt (*Verticillium albo-atrum*), anthracnose (*Colletotrichum meliloti*), aphanomyces root rot (*Aphanomyces euteiches*), common leaf spot (*Pseudopeziza medicaginis*), downy mildew (*Peronospora trifoliorum*), fusarium wilt (*Fusarium oxysporum* f. sp. *medicaginis*), phytophthora root rot (*Phytophthora megasperma* Drechs. f. sp. *medicaginis*), and stemphylium leaf spot (*Stemphylium botryosum*). Genetic progress has been achieved for most of them, leading to the release of cultivars with high levels of resistance to multiple diseases (e.g., see the USA alfalfa cultivar list at http://www.alfalfa.org). For some diseases, several pathogenic strains have been identified during germplasm screening and variety selection.

The genetic control of resistance to most alfalfa diseases and pests has not been elucidated in detail, though anthracnose is an exception. Mackie et al. (2007) have identified a major quantitative trait locus (QTL) for resistance to races 1 and 4 at the end of chromosome 8, and two significant QTL for resistance to race 2 on chromosome 4, along with a number of QTL with smaller effects. Resistance to anthracnose has been located in *M. truncatula* due to the high level of synteny with alfalfa (Ameline-Torregrosa et al. 2008). One resistance gene, RTC1, has

been mapped on the genetic and physical maps of *M. truncatula* and cloned (Yang et al. 2007). RTC1 is a member of the Toll-interleukin-1 receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant R genes and confers broad spectrum resistance to anthracnose in alfalfa (Yang et al. 2008). Numerous improved germplasms have been released, such as NM-9D11A-AN3 (Ray et al. 2000) and breeding resistant cultivars has been successful (Irwin et al. 2006).

Bacteria. Bacterial wilt is the most important bacterial disease of alfalfa, and although it does not occur throughout the worldwide range of alfalfa cultivation, it has a major economic impact in North America. *Clavibacter michiganensis*, one causative agent, may be transmitted through alfalfa seed, requiring adequate PCR detection systems to be developed and implemented. Standardized screening tests of field or greenhouse grown seedling plants are well established (http://naaic.org/stdtests/index.html). *Ralstonia solanacearum* also induces bacterial wilt in alfalfa, as well as in more than 200 other plant species. This pathogen showed a strain × genotype interaction among *M. truncatula* accessions. A major QTL for resistance to this bacterial wilt was identified (Vailleau et al. 2007). The results for these two pathogens in *M. truncatula* open prospects for identifying resistance in alfalfa. Resistance may likely become increasingly important because of climate change, which may increase the occurrence and severity of this disease in regions where it had presently no or little impact.

7 Breeding Methods and Specific Techniques

7.1 General Breeding Methods

Selection methodologies applied to cultivated alfalfa, a typical autotetraploid species, are complicated by marked inbreeding effects, natural allogamy, and by abundant small, perfect flowers, which are difficult to handle. As a consequence of the reproductive system and of the constraints of the floral structure, hybrid production is difficult; consequently, the large majority of the alfalfa cultivars are populations in random mating equilibrium whose identity and agronomical values are maintained by the constancy of gene and genotypic frequencies across generations (Piano and Veronesi, 1996). Allogamy, effected by bee pollination, results in substantial phenotypic flexibility in cultivars due to the presence of a large number of different genotypes in any given population. Natural selection among these genotypes occurs in the cultivation environments, results in genetic drift, a potentially serious problem during seed production.

Alfalfa breeding has progressed from ecotype selection through mass selection, phenotypic recurrent selection, and strain building, with a limited amount of backcross breeding, all of which lead to synthetic cultivar development (Rumbaugh et al. 1988). The large majority of the modern alfalfa cultivars are still based on synthetic breeding approaches, with progeny testing being used in some commercial programs. For quantitatively inherited traits of low to moderate heritability,

progeny testing is decidedly superior to phenotypic recurrent selection (Fehr 1987). The comparative advantage or disadvantage of different methods of progeny test selection has been assessed (e.g., Rowe and Hill 1985; Casler and Brummer 2008).

Breeding procedures based on single plant selection under spaced plant conditions do not consider intraspecific competition as the basis of the plant performance in a dense stand such as an alfalfa meadow (Rotili and Zannone 1975; Veronesi and Lorenzetti 1983). Even if this problem has been stressed several times by both American and European researchers, practical alfalfa breeding is still conducted under spaced plant conditions with good results for Mendelian traits such as disease resistances, but with inconsistent results with respect to quantitative traits such as forage or seed yield.

It is not possible to "fix" an interesting alfalfa genotype in a synthetic cultivar. Even if the breeder were able to select genotypes bearing superior gene combinations, the plants would need to be intercrossed to produce synthetic cultivars, which mainly takes advantage of average effects of the genes (additive effects), although a limited amount of heterotic effects may remain in the certified seed generation.

7.2 Genetics of Autotetraploids, Heterosis, and Hybrid Production

Alfalfa breeders and researchers have attempted to develop inbred lines to be used as parents of hybrids since the 1930s, but because of severe inbreeding depression, inbred lines are nearly impossible to develop. Carnahan (1960) suggested that heterosis in polysomic polyploids was more complicated than in diploids in that four different alleles at a locus were needed for maximum heterozygosity. Shortly thereafter, Demarly (1963) published theoretical models and practical results supporting the importance of tetra-allelism (or four different chromosome segments – linkats, see later) in heterosis expression in alfalfa.

Most genetic knowledge about heterosis in alfalfa has been inferred from inbreeding studies. Generally, inbreeding depression is considered as the converse of heterosis, although the genetic mechanism could be somewhat different (Ritland 1996). Inbreeding tetraploid alfalfa results in more substantial depression in vigor (yield) than might be expected based solely on the decrease in heterozygosity. This severe inbreeding depression is likely due to the loss of multiple-allele interactions (Busbice and Wilsie 1966), the loss of favorable dominant alleles at different loci that are linked in repulsion phase, and/or the loss of desirable epistatic combinations of alleles (Bingham et al. 1994; Woodfield and Bingham 1995; Kimbeng and Bingham 1998). The latter two explanations consider the genome to consist of blocks of linked loci, called linkats (Demarly 1979). For convenience, we use "allele" interchangeably with "linkat" as the actual genetic basis of heterosis remains unknown.

Tetrasomic inheritance patterns typical of an autotetraploid species are quite complex and create problems in breeding. In a diploid species ($2n = 2x$) only a

single heterozygous genotype is possible (i.e., a_1a_2); in other words, the heterozygote shows just one interaction among alleles at the same locus. In an autotetraploid ($2n = 4x$), a single locus with only two alleles has three different heterozygous genotypes in addition to the two homozygous classes, *viz*., $a_1a_1a_1a_1$, $a_1a_1a_1a_2$, $a_1a_1a_2a_2$, $a_1a_2a_2a_2$, and $a_2a_2a_2a_2$. But, adding further complexity, multiple alleles can be present at a given locus in polyploids, meaning that monoallelic ($a_1a_1a_1a_1$), diallelic simplex, (e.g., $a_1a_1a_1a_2$ or $a_1a_1a_1a_3$), diallelic duplex (e.g., $a_1a_1a_2a_2$ or $a_1a_1a_3a_3$), triallelic (e.g., $a_1a_1a_2a_3$), and tetraallelic ($a_1a_2a_3a_4$) genotypes are possible at a given locus (or among a set of linkats).

As a consequence, with complete dominance a specific trait can be expressed at the same level in different genotypes, with partial dominance an expression gradient due to a dosage effect can be observed. The maximum heterozygosity is reached with the tetraallelic situation, in which each allele or each linkat is different and which is able to produce 6 first-order interaction (a_1a_2, a_1a_3, a_1a_4, a_2a_3, a_2a_4, a_3a_4), 4 second-order interactions ($a_1a_2a_3$, $a_1a_2a_4$, $a_1a_3a_4$, $a_2a_3a_4$), and 1 third-order interaction ($a_1a_2a_3a_4$) for a total of 11 potential intraallelic (intralocus) interactions. Furthermore, the effects of interallelic (epistatic) interactions among alleles at different loci must be considered; these effects are very complex and can produce strong distortions during the breeding procedures, being able to confound the real value of single genes.

As a consequence of this complexity, many deleterious recessive alleles can be maintained in populations and consequently, inbreeding depression is very strong in alfalfa, especially for fertility and vigor (Busbice and Wilsie 1966; Rotili and Zannone 1977). Developing highly homozygous lines, ideal for hybrid breeding approaches, is difficult. Yet even if inbreds can be developed, the recovery or maximization of heterosis upon crossing is not attained in the F_1 generation. The complexity of heterozygosity in species with polysomic inheritance like alfalfa means that the maximum level of heterotic vigor in progeny of inbred parents occurs in double cross hybrids or potentially even more complex hybrids (Bingham et al. 1994). This phenomenon, known as progressive heterosis, has been experimentally demonstrated in alfalfa (Groose et al. 1989; Li and Brummer 2009) and is likely the consequence of tri- and tetra-allelic loci which can only be expressed in double crosses or later generations (Table 2). However, if parents are unrelated and non-inbred, single cross hybrids may maximize heterosis (Dudley 1964). Thus, in alfalfa it is not possible to exploit heterosis with the same breeding procedures utilized in diploid species such as corn (*Zea mays* L.).

As reported by Bingham (1980), double cross hybrid production in alfalfa was proposed long before the awareness of the advantages it offered in maximizing hybrid vigor (Tysdal et al. 1942; Tysdal and Kiesselbach 1944; Bolton 1948). Commercially produced double crosses would be produced without pollination control and thus, are a mixture of chance double crosses (i.e., crosses between individuals derived from the two different single cross hybrids) and hybrids between single cross sibs.

A reasonably suitable cytoplasmic male sterility system has been described (Viands et al. 1988), but nevertheless, producing commercial quantities of hybrid

Table 2 Theoretical genetic structures in alfalfa populations obtained from inbred lines, single or double crosses, under the assumptions that frequency of different alleles per locus was equal and that single and double crosses were made by mating unrelated parents (modified from Bingham, 1980)

Parents	Cross population	Parental genotypes	Genetic composition of the cross population
Inbred lines A, B	Single crosses (A×B)	100% monoallelic $a_1a_1a_1a_1 \times a_2a_2a_2a_2$	100% diallelic duplex $a_1a_1a_2a_2$
Single crosses (A × B) (C × D)	Double cross (A×B)×(C×D)	100% diallelic duplex $a_1a_1a_2a_2 \times a_3a_3a_4a_4$	11.1% diallelic duplex $a_1a_1a_3a_3$ $a_1a_1a_4a_4$ $a_2a_2a_3a_3$ $a_2a_2a_4a_4$ 44.4% triallelic $a_1a_1a_3a_4$ $a_1a_2a_3a_3$ $a_2a_2a_3a_4$ $a_1a_2a_4a_4$ 44.4% tetraallelic $a_1a_2a_3a_4$
Double crosses [(A×B)×(C×D)] [(E×F)×(G×H)]	Between double crosses [(A×B)×(C×D)] × [(E×F)×(G×H)]	11.1% diallelic duplex 44.4% triallelic 44.4% tetraallelic	1.2% diallelic 19.8% triallelic 79.8% tetraallelic
Random mating equilibrium[1]			1.6% monoallelic 18.7% diallelic simplex 14.1% diallelic duplex 56.2% triallelic 9.4% tetraallelic

[1]From Dunbier (1974) based on equal frequencies of four alleles.

seed on male-sterile plants is difficult, due to limited cross pollination by insects, who avoid plants without pollen.

More recently, Rosellini et al. (2003) found alfalfa mutants with a high level of female sterility (Figure 7) which, in theory, could be used as pollen sources in alfalfa true hybrid production, but their practical use is not possible at the moment. Hence, the actual situation vis-a-vis hybrid alfalfa is almost the same as in the past and real double cross hybrids from controlled pollination are not yet economical.

Nevertheless, since the beginning of the 21st century, some "hybrid" cultivars produced based on a male sterility system entered the alfalfa seed market (Sun et al. 2004). This method does not produce true single cross hybrids like those in maize or canola, but rather results in a narrow-based population which contains more than 75% hybrid plants derived from the hybridization between two different "lines." Data from USA (Wiersma 2001) show that one of them consistently ranked in the

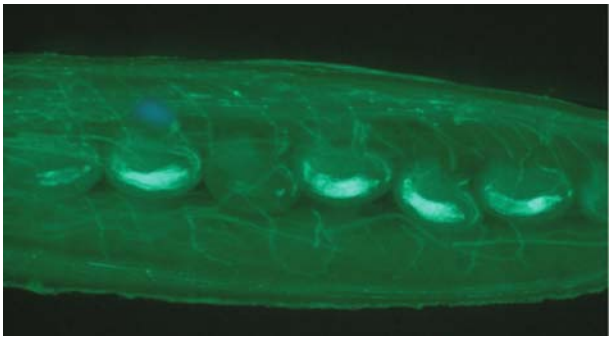

Fig. 7 Photo micrograph of alfalfa ovules at anthesis stained with aniline blue (Photo D. Rosellini)

top 10% of each of 25 different test environments, indicating that it was very stable and had improved yield performance as compared to some non-hybrid cultivars on the market. As a consequence, advances in alfalfa hybrid breeding seem to offer potential for yield improvement.

7.3 Chance or Semi-Hybrids

Producing hybrids between genetically non-related parental populations could lead to a high level of tetraallelic loci at the cultivar level. Therefore, a potentially effective way to improve yield in the future is to capitalize on nonadditive gene action by harnessing heterosis through the use of divergent populations and semi- or chance hybrids (Brummer 1999; Scotti and Brummer 2009). A semi-hybrid breeding strategy based on population crosses would avoid the need for inbred lines and would capture some heterosis (Brummer 1999). Population hybrids between *M. sativa* subsp. *sativa* and *M. sativa* subsp. *falcata* show heterosis for biomass production (Sriwatanapongse and Wilsie 1968; Riday and Brummer 2002a; Riday and Brummer 2005), suggesting possible heterotic groups within alfalfa germplasm.

The striking differences in morphology, geographical distribution, and phenology between *M. sativa* subsp. *sativa* and *M. sativa* subsp. *falcata* suggest that favorable complementary gene interactions may exist between the two groups, explaining the observed heterosis. If this were the case, larger inbreeding depression in F_2 or later generations should also be observed, if the hybrid were advanced, causing greater biomass yield loss. In particular, if favorable (and different) epistatic allele combinations exist in each heterotic group, additional generations would lead to disruptions of the coadapted gene complexes leading to greater yield loss than similar advanced generations in intrasubspecific crosses. If intersubspecific crosses have poor performance in advanced generations, then hybrid populations should not be used for future recurrent selection. A better approach for germplasm maintenance and for

repeated capitalization on heterosis would be to keep the parental germplasms separate and only produce hybrids between them as the final step in the breeding process, as suggested by Brummer (1999).

Experimental data suggest that selection within each parental population followed by intercrossing to produce intersubspecific hybrids may be a better way to improve *M. sativa* biomass using *M. sativa* subsp. *falcata* germplasm than advancing hybrids into a recurrent selection program (Li and Brummer 2009). Future breeding development will show if this approach is worthwhile in alfalfa.

Despite its obvious value for yield, *M. sativa* subsp. *falcata* has deleterious characteristics, such as slower growth and more decumbent growth than subsp. *sativa*, characteristics that undermine the value of intersubspecific hybrids (Riday and Brummer 2002b). Research into other germplasm crosses have shown that the nondormant Peruvian germplasm, in particular, offers potential to realize heterotic yield gains, at least in certain crosses (Segovia-Lerma et al. 2004, Madrill et al. 2008). Crosses among other divergent germplasms can also show heterosis (Bhandari et al. 2007), although determining exactly which germplasms to hybridize probably will require empirical testing. Finally, some hybrids among diverse germplasm sources, such as semidormant midwestern USA germplasm and populations derived from nondormant cultivars, do not express any useful level of heterosis (Şakiroğlu and Brummer 2007). The somewhat arbitrary combining abilities of different germplasms suggests that a more productive approach to developing heterotic groups is to create them de novo by reciprocal recurrent selection (RRS; Fehr 1987) than to simply rely on past genetic divergence. Although to our knowledge no significant attempt at RRS has been attempted in alfalfa, the potential for that method to produce high yielding semi-hybrid cultivars seems high.

7.4 Analytic Breeding

The breeding approach known as analytic breeding (Chase 1964) involves the reduction of the ploidy level from 4x to 2x by haploidy (Dunbier 1974; Bingham and Mc Coy 1979), breeding at the 2x level, and then returning to the 4x level via bilateral sexual tetraploidization, making use of male and female unreduced (2n) gametes, or via an unreduced gamete in one parent crossed to a tetraploid plant producing normal gametes (Figure 8).

This process offers an alternative to normal sexual reproduction to attain heterozygosity at or near the maximum: hybridization of unreduced gametes arising from unrelated, heterozygous male and female diploid plants (Bingham 1980). If high frequencies of 2n gametes and sufficient seed could be produced, this method could produce genetically uniform tetraploid cultivars showing a high level of tri and tetraallelic loci, making possible the commercial exploitation of the heterosis at the autotetraploid level. In particular, 2n gametes produced by first division restitution (FDR) mechanisms or an equivalent process can be effective breeding tools in polysomic polyploids, transmitting all the heterozygosity from the centromere to the first crossover and half of that between the first and the second crossover (Mendiburu

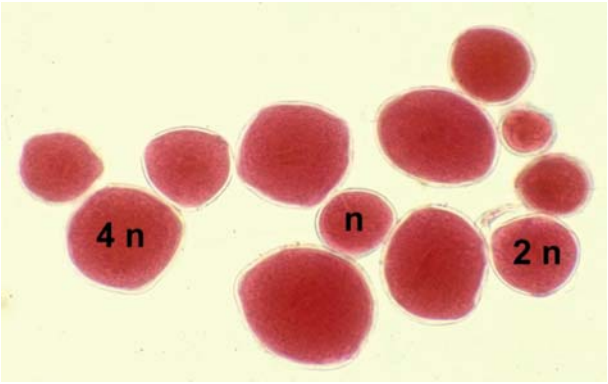

Fig. 8 n, 2n (unreduced), and 4n (jumbo) pollen grains produced in diploid alfalfa mutants (Photo Department of Applied Biology, University of Perugia, Italy)

1971; Mok and Peloquin 1975). Alfalfa typically has only one chiasma per bivalent; it is estimated that approximately 80% of the heterogosity is transmitted from parent to offspring via FDR 2n gametes.

Furthermore, 2n gametes are widely diffused in the *M. sativa* complex (Veronesi et al. 1986). Theoretically, maximum heterozygosity in alfalfa could be achieved with an analytic breeding approach through the exploitation of mutants characterized by a concomitant absence of crossing over and FDR (Figure 9).

Of course, the problem with the scheme is that meiotic mutants would interfere with advanced generation seed production at the tetraploid level, so unless the diploids were able to produce commercial quantities of seed, this method is unlikely to be viable. Nevertheless, analytic breeding could be an integral part of a traditional recurrent selection breeding program. The ability to purge undesirable alleles is much simpler at the diploid level, and the possibility of fixing advantageous alleles much higher. Re-tetraploidization could be conducted by crossing 2n egg or pollen producing diploids with elite tetraploid genotypes to directly incorporate diploid germplasm into tetraploid breeding populations. Because large quantities of seed are not necessary, highly penetrant meiotic mutants will not need to be used, thereby not affecting ultimate seed production. Finally, although chromosome doubling by colchicine or similar chemicals introduces a level of inbreeding, that may not be significant in a recurrent selection program where plants will be intercrossed and selected for multiple generations.

8 Integration of New Technologies in Breeding Programs

Genomic technologies present opportunities to improve alfalfa cultivars more quickly and efficiently (Brummer 2004). Despite an increase in basic and applied genetic knowledge over the past several decades, alfalfa cultivars are largely

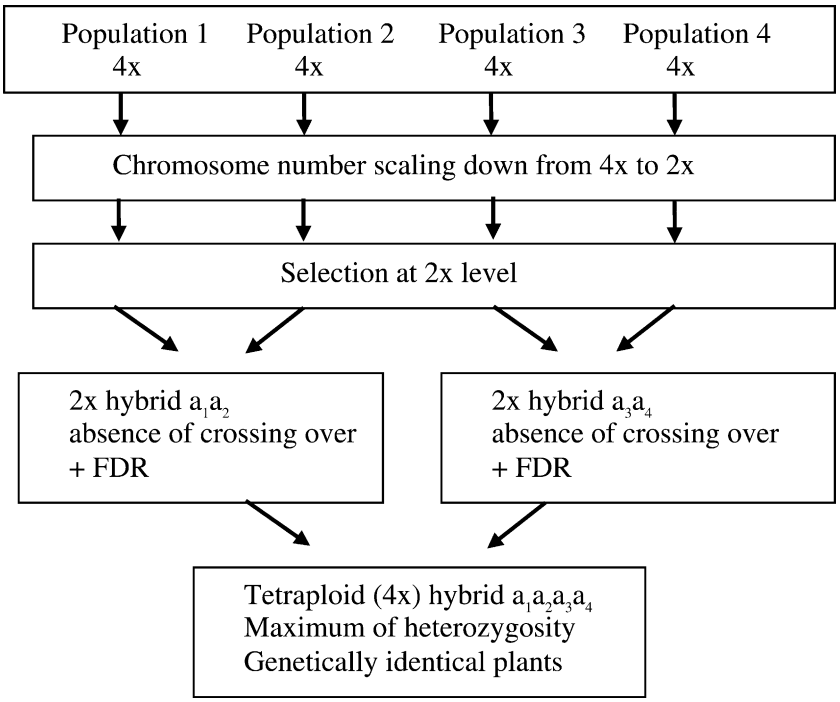

Fig. 9 Analytic breeding scheme and possible use of 2n gametes in double cross hybrid production (modified from Piano and Veronesi 1996)

produced by conventional breeding methods. The efficiency of alfalfa breeding programs could be augmented by direct selection at the genotypic level using molecular markers co-segregating with the plant genes of interest (Barcaccia et al. 2003). However, the optimism generated by these technologies needs to be tempered by the complexities and peculiarities of alfalfa biology, as described above. A consequence of synthetic cultivars is that fixing markers or loci or transgenes in a cultivar is exceedingly difficult, limiting the application of genomic technologies, or at least requiring that they be applied in ways different from those used in inbred line or hybrid cultivar development.

8.1 Genetic Diversity Assessment

Genetic markers have been used to assess genetic diversity within and among alfalfa populations (Brummer et al. 1991; Kidwell et al. 1994a; Crochemore et al. 1996; Ghérardi et al. 1998; Pupilli et al. 2000; Segovia-Lerma et al. 2003; Zaccardelli et al. 2003; Maureira et al. 2004; Flajoulot et al. 2005; Vandemark et al. 2006; Ariss and Vandemark 2007; Greene et al. 2008; Şakiroğlu et al. 2009a). The major points from

these experiments are that (1) alfalfa populations – both wild and cultivated – are highly diverse and (2) most genetic variation in alfalfa resides within populations.

Considerable effort has been expended in attempting to differentiate among the nine historical germplasms brought into the USA, as enumerated by Barnes et al. (1977). In general, these experiments have shown that subsp. *falcata* is distinct from subsp. *sativa* (Kidwell et al. 1994a; Musial et al. 2002; Segovia-Lerma et al. 2003; Vandemark et al. 2006; Ariss and Vandemark 2007). How well these original sources represent current alfalfa germplasm is questionable, given that repeated introductions of germplasm from abroad into the USA have occurred throughout the 20th and 21st centuries with subsequent mixing among all sources.

A large study of semi-dormant and nondormant germplasm placed all the historical populations within a larger group that included only the very nondormant cultivars (Ariss and Vandemark 2007). Given that the historical germplasms span the alfalfa dormancy range, these results suggest that they are not representative of current breeding germplasm, but that they may be worth investigating further because of that differentiation.

The Peruvian germplasm – shown above to produce heterosis in certain hybrid combinations – was noted to be distinct from other *sativa* material (Kidwell et al. 1994a) and a subsequent examination showed that Peruvian, falcata, and a group of three modern USA cultivars were clearly distinct from each other (Mauriera et al. 2004). The three cultivars were rather similar in overall genome composition based on a Bayesian analysis of marker profiles and hence were more closely related to one another than any of the three was to the Peruvian or falcata germplasm.

Because alfalfa populations are heterogeneous, analysis of several plants per population is necessary to accurately represent the variation present. Few diagnostic markers have been identified among populations in any marker study to date; instead, differences among populations are manifested in different allele frequencies. Therefore, bulking of individuals within accessions bears the risk of eliminating the source of much variation among populations unless the allele frequency can be quantitatively estimated from the resulting assay. Bulking has been done in numerous experiments and the results show that populations can still be separated successfully (Segovia-Lerma et al. 2003; Ariss and Vandemark 2007). However, variation can be observed among bulks of a given cultivar. Ariss and Vandemark (2007) attribute this to within population genetic variation, but it seems odd that bulks of 20 plants would diverge greatly for other than methodological reasons.

One of the uses of genetic marker diversity analysis is to identify cultivars, or to distinguish among cultivars, for purposes of plant variety protection. Although markers have proven useful in general terms to distinguish among cultivars (e.g., Brummer et al. 1991; Ariss and Vandemark 2007), they have not been able to differentiate among closely related – but morphologically unique – cultivars derived from a single breeding program, even though that program routinely incorporated additional germplasm (Flajoulot et al. 2005).

Despite the research that has been done on genetic diversity in alfalfa, surprisingly little systematic investigation of alfalfa germplasm has been attempted. The

most thorough published experiment to date investigated mitochondrial diversity in diploid and tetraploid germplasm (Muller et al. 2003). Their conclusions show (1) no differentiation between wild diploid subsp. *caerulea* and wild tetraploid subsp. *sativa*, confirming that they are the same group represented at two ploidy levels, (2) unique mitotypes of subsp. *falcata* in the eastern part of its distribution, (3) gene flow between subsp. *sativa* and *falcata* is widespread, particularly in western Europe, and (4) less mitochondrial diversity in cultivated than wild gene pools. Gene flow between cultivated and wild alfalfa has been documented in several cases with molecular markers (Jenczewski et al. 1999a, b; Muller et al. 2003; Greene et al. 2008). Some wild populations from Kazakhstan appear to have little to no introgression from cultivated forms, but others show evidence of admixture (Greene et al. 2008). Interestingly, Greene et al. (2008) observed similar numbers of alleles and polymorphic loci in both Russian cultivars and Kazakh wild populations.

A recently conducted analysis of 120 diploid *M. sativa* accessions using 89 SSR markers clearly differentiated subsp. *falcata* from subsp. *caerulea* and identified hybrid subsp. *hemicycla* (Şakiroğlu et al. 2009a). In addition, two populations were identified within each of the main subspecies, with *caerulea* being differentiated into a northern and southern population and *falcata* bifurcating into a population that appears to be adapted to upland, dry habitats and a second from lowland, littoral environments. These results suggest relatively limited gene flow between the two main diploid subspecies, except in clear regions of sympatry (Şakiroğlu et al. 2009b). Phenotypic evaluation of biomass production, cell wall composition, and agronomic traits did not separate the germplasm into discrete groups as did the marker analysis, suggesting that desirable alleles may reside in all germplasm sources (Şakiroğlu et al. 2009c). Thus, diploid germplasm could be selected for future incorporation into cultivated alfalfa based on diverse origins, in order to maximize genetic variation and to include more alleles, using the analytic breeding scheme described above.

Several experiments have attempted to associate marker diversity with population or hybrid performance. In one experiment, genetic distance and biomass yield were correlated at the tetraploid, but not at the diploid level (Kidwell et al. 1994b). This result suggested that maximizing marker-based diversity among parents could result in higher yielding progeny populations. Unfortunately, this hypothesis was not supported in a subsequent examination of populations selected based on molecular marker similarity or dissimilarity using commercially elite germplasm, as genetic distance and yield were unrelated (Kidwell et al. 1999). The reason for the lack of association in the second experiment may have been due to linkage equilibrium in the population and/or to the use of markers that were not linked to genes associated with yield.

Similar contradictory results have been found in relation to heterosis and inbreeding. The genetic distance of parents was not related to their levels of heterosis for biomass yield (Riday et al. 2003), again possibly because a targeted set of markers was not used (it was not, and still is not, available!). Heterozygosity was unrelated to the vigor of S_2 plants within families (Scotti, et al. 2000). However, another experiment has shown that parental genetic diversity is highly correlated with not only

biomass yield, but also with high parent heterosis and with specific combining ability (Scotti and Brummer 2009). Apart from using different germplasm, the causes of these divergent results could be due to the markers chosen. In any case, a clear relationship between particular markers and biomass yield and/or yield heterosis has not been determined, but if it were, it would undoubtedly be useful to improve yield.

8.2 Genetic Mapping

Genetic mapping in alfalfa initially focused on diploid germplasm, and a number of diploid maps have been developed in the past 15+ years (Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994; Tavoletti et al. 1996a; Brouwer and Osborn 1997; Barcaccia et al. 1999; Kaló et al. 2000; Tavoletti et al. 2000; Porceddu et al. 2002; Sledge et al. 2002). Five maps in tetraploid alfalfa, all based on F_1 populations, have been published to date (Brouwer and Osborn 1999; Julier et al. 2003b; Sledge et al. 2005; Musial et al. 2005; Robins et al. 2007a). Initial maps were based on restriction fragment length polymorphism (RFLP) and random PCR-based markers such as randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP). Subsequently, most mapping has been conducted using simple sequence repeat (SSR) markers. Single nucleotide polymorphisms (SNP) will probably be the marker of choice in the near future as they keep being developed, particularly those present within candidate genes for agronomically important traits.

Initial maps were not based on a reference genome, and consequently, due to the fact that they have few markers in common among them, cross-referencing is not generally feasible (Brummer et al. 2001). However, research on *M. truncatula*, particularly the sequencing of its euchromatic genome space, has provided a basis for linkage group nomenclature. The chromosomes of alfalfa were aligned to *M. truncatula* by Choi et al. (2004) using cleaved amplified polymorphic sequence (CAPS) markers and Kaló et al. (2000). Large numbers of SSR markers have been developed directly from *M. truncatula* sequences, particularly those of expressed sequence tags (ESTs) (Baquerizo-Audiot et al. 2001; Julier et al. 2003b; Eujayl et al. 2004), which has further facilitated the assignment of alfalfa linkage groups in all recent genetic maps to *M. truncatula* chromosomes. Nevertheless, no common reference genetic map exists for alfalfa at the current time, so many markers have only been localized in a single population.

Several traits have been mapped in diploid alfalfa populations, including the unifoliolate leaf/cauliflower head (*uni*) mutation (Brouwer and Osborn 1997), jumbo pollen (*jp*) and multinucleate microspore formation (Tavoletti et al. 2000), and sticky leaves (*stl*), dwarf phenotypes, flower color, and genes associated with nitrogen fixation and other traits (Kiss et al. 1993; Kaló et al. 2000). Mapping was performed in a half-tetrad analysis to identify the mode of 2n egg formation of PG-F9, a diploid genotype displaying second division restitution (Tavoletti et al. 1996b). Aluminum tolerance is the only agronomically important trait that has been mapped at the diploid level (Sledge et al. 2002; Narasimhamoorthy et al. 2007).

Mapping on the tetraploid level has been focused on agronomic traits, including mapping quantitative trait loci (QTL) for biomass yield and morphological traits (Musial et al. 2006; Robins et al. 2007a, 2007b), persistence (Robins et al. 2008); winter hardiness (Brouwer et al. 2000; Alarcón-Zúñiga et al. 2004), resistance to *Phytophthora medicaginis* (Musial et al. 2005), resistance to *Stagonospora meliloti* (Musial et al. 2007), resistance to *Colletotrichum trifolii* (Mackie et al. 2007), and a non-nodulating phenotype (Endre et al. 2002). In addition to these published accounts, we know of work to map aluminum and acid-soil tolerance, cell wall composition, and drought tolerance, among other traits that is ongoing around the world. Finally, mapping in *M. truncatula* offers some potential useful targets for application to alfalfa, including mapping of morphogenic traits (Julier et al. 2007), flowering time (Pierre et al. 2008), and resistance to *Aphanomyces euteiches* (Pilet-Nayel et al. 2009). In these experiments, typically many QTL of relatively small effects are identified. Given the generally small population sizes of these experiments, QTL effects are likely biased. The primary need at the current time is for additional experiments in different genetic backgrounds to be conducted, to help generate a clearer view of genomic regions important for various traits.

As shown above, mapping of most agronomically important complex traits, such as biomass yield, cell wall composition, or disease resistances, has not been attempted in diploid alfalfa. This is somewhat unfortunate, as the simplified segregation ratios and superior map resolution in diploids would make mapping at that level considerably easier than in tetraploids. Mapping in tetrasomic tetraploids is complicated by the fact that four homologous chromosomes need to be mapped simultaneously, necessitating a considerably larger number of markers to fully saturate each chromosome than is needed in diploids (Figure 10). Thus, the density of the consensus group hides the fact that many areas on the individual homologues are devoid of markers. Further, the dosage of alleles is typically not known, introducing considerable uncertainty into the true constitution of each genotype in a population. Software for map construction and QTL identification has been developed (Hackett et al. 2007), which helps with the mechanics of mapping but does not address the issues alluded to above.

Mapping in alfalfa would benefit from several considerations. First, the development of a reference diploid mapping population on which the majority of widely used markers could be incorporated and which is tied explicitly to the *M. truncatula* genome sequence (which should be essentially finalized in 2009) would provide a robust framework on which to build all future mapping projects. A framework map would facilitate the comparison of QTL map locations, something that at the current time is difficult or impossible to accomplish. Second, additional mapping projects at the diploid level should be initiated, with the goal of developing a "first-pass" view of the genetic architecture of some important agronomic traits. Of course, the genetic control of complex traits may not be identical across ploidy levels, as has been shown for gene expression profiling in yeast (Galitski et al. 1999), and for combining ability differences in alfalfa (Groose et al. 1988). Nevertheless, focusing more efforts on diploids would enhance our ability to identify key QTL, localize them to small genomic regions, and facilitate their cloning or use of closely linked markers in selection.

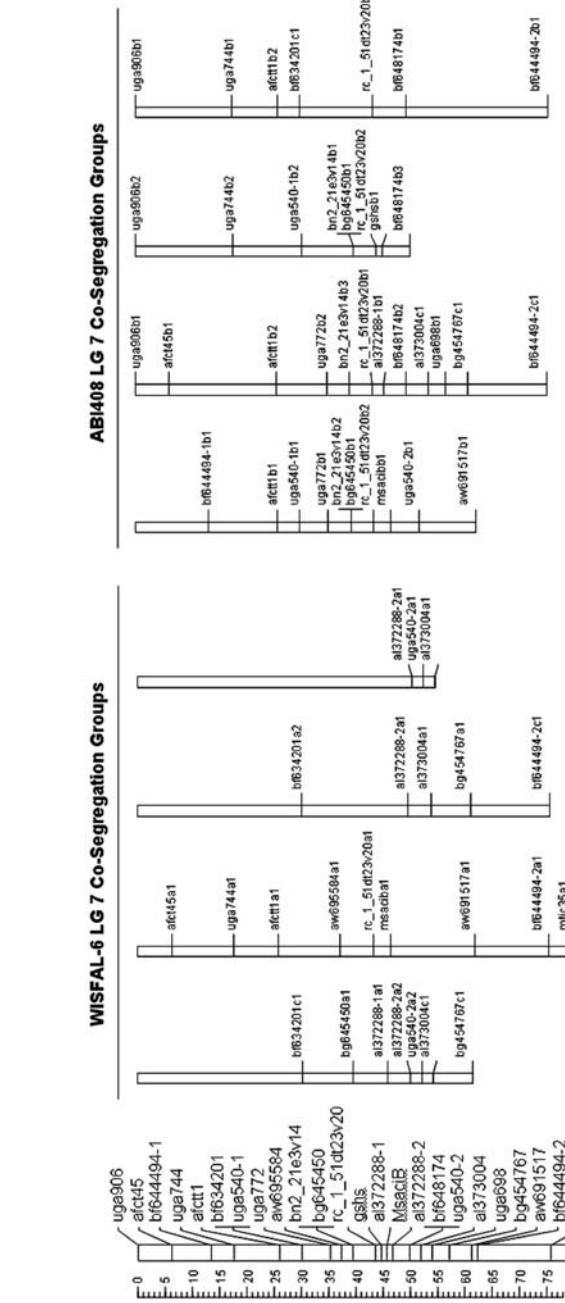

Fig. 10 Example of tetraploid linkage maps (Robins et al. 2007a). In this F_1 population, tetraploid map was used to create a consensus map (*left*) based on markers assigned to the four homologous chromosomes from each parent (WISFAL-6 and ABI-408)

An alternative to using biparental mapping populations to detect QTL is the use of association mapping, whereby markers are genotyped on individuals within a breeding population, which are also phenotyped. Association mapping is conceptually better suited to alfalfa breeding, which results in synthetic populations for commercialization, than are biparental populations because the latter only account for a small amount of the allelic variation within the overall population. The primary limitation to association mapping is the requirement for a very large number of markers unless residual linkage disequilibrium in the population is substantial – an unlikely proposition for many breeding populations. Our preliminary estimation of linkage disequilibrium in one breeding population suggests that it is quite low, perhaps one Mbp or less (Wei and Brummer, unpublished).

Association mapping has been attempted in one experiment (Mauriera-Butler et al. 2007). In this experiment, individuals from a population developed from a cross of *M. sativa* subsp. *falcata* and the Peruvian germplasm were genotyped with 39 genetic markers (RFLP and SSR) and test crossed to two elite clones derived from different commercial cultivars. Testcross yield and fall growth data were collected and used for association with markers. Interestingly, even with this low density of markers, multiple regression models explaining ~25% of phenotypic variation for yield and 34% for fall growth, lending credence to the idea that relatively few markers can be usefully applied to mapping in alfalfa breeding programs.

8.3 Using Markers and Mapping in Breeding

Perhaps the bigger question with genetic markers and mapping is how the results will be put to practical use in cultivar development programs. Two primary avenues seem most worthy of in depth investigation (Bernardo 2008).

8.3.1 Marker-Assisted Introgression

Marker-assisted introgression of QTL is the most obvious use of markers. Genetic mapping is used to identify QTL, and then, using linked markers, the desired QTL allele is backcrossed into elite cultivars. If the QTL allele has a large effect on the phenotype, then introgression will likely be usefully undertaken.

Two issues present themselves with this method, which has not been used directly in alfalfa breeding to date. The first impediment to using marker-assisted introgression is the initial identification of prospective QTL. Mapping in tetraploids is complex, as noted above, due to the presence of four homologous chromosomes in each plant. Thus, even though QTL may be detected, locating them precisely is quite difficult without very large population sizes and an accompanying high density genetic linkage map. Even then, the precision will likely be low, particularly compared to a diploid map, and ensuring that the markers used for introgression are linked in coupling with the QTL allele of interest is difficult.

The second impediment arises as a direct result of the first, in that once markers have been identified that are correctly linked to the right QTL allele, backcrossing needs to be done to several individuals within the population of interest to maintain sufficient genetic variation to avoid inbreeding depression. Depending on the population into which the QTL allele is introgressed, further backcrossing may be necessary. Ultimately, in order for the allele to be useful, it will need to be selected repeatedly in future breeding to increase its frequency in the population. Obviously, not many QTL can be selected simultaneously in a population before segregation ratios become unwieldy.

8.3.2 Marker-Assisted Selection

The canonical "marker-assisted selection" scenario envisaged by many plant breeders in the early days of molecular marker mapping – identify markers linked to QTL and then use those markers for future breeding – does not integrate well into recurrent selection schemes used in essentially all current alfalfa breeding programs. The main problems are (1) that as markers are near fixation, the effect of the allele declines, necessitating re-mapping to identify other QTL and (2) that selection for many QTL simultaneously quickly requires population sizes that are unacceptably large (Brummer and Casler 2008). If a QTL effect is large enough, then the marker-assisted introgression approach described above works best (Bernardo 2008). Unlike the major arable crops in which pure lines function either as the cultivar per se or as the means to produce a hybrid cultivar, alfalfa is commercialized as synthetic populations. Therefore, markers – and QTL – will not be easily fixed in a population. Indeed, the identification of QTL using bi-parental populations may not even identify the most important QTL in the population. The application of markers in alfalfa, therefore, likely has more in common with animal breeding methodologies in which whole genome selection holds the most promise to improve genetic gain.

8.3.3 Whole Genome Selection

Whole genome selection, unlike other marker-assisted schemes, is a "black box" method in which knowledge of QTL is not necessary. The method for whole genome selection is as follows (Bernardo and Yu 2007): markers distributed throughout the genome are genotyped on individuals that are also phenotyped (or their progeny are phenotyped). Each marker is then assigned a breeding value based on the phenotypic data, and in subsequent generations of selection and intercrossing, individuals are selected based on their "net worth" obtained by summation of marker breeding values across all markers.

Whole genome selection appears to be the marker method most in line with current alfalfa breeding methods, complementing a recurrent selection program. The main element of importance for the method to work is the extent of linkage disequilibrium (LD) in the population. In large, diverse breeding populations, linkage disequilibrium may only extend short distances; if this is the case, then thousands

(or tens of thousands) of markers are necessary to saturate the genome, making the method prohibitively expensive. However, in narrow-based breeding populations, this may not be a problem, as bottlenecked populations may have LD that extends far enough that only a few hundred markers may be needed to cover the genome. The alternative method, if LD is not extensive, is to only use markers in or closely linked to candidate genes that may be involved with the trait. While marker numbers may initially limit the utility of whole genome selection in alfalfa, extensive sequence-based polymorphisms (SNPs) are likely to be available in the near future as DNA sequencing costs continue to fall.

8.4 Gene Expression and Metabolomics

Relatively little work has been reported on gene expression experiments in alfalfa beyond the gene-by-gene analysis of transgene expression. Affymetrix arrays consisting of ~50,000 genes from *M. truncatula*, ~2,000 from *M. sativa*, and ~8,000 from *Sinorhizobium meliloti* have been developed and used in alfalfa (Tesfaye et al. 2006; Li et al. 2009). In both experiments, ~50% of the probe sets on the array were expressed in alfalfa, indicating that arrays developed from *M. truncatula* are useful for alfalfa. The experiment of Li et al. (2009) examined gene expression in alfalfa hybrids. While most genes showed additive expression, a higher proportion showed non-additive expression in two hybrids expressing heterosis for biomass yield. In addition, a higher proportion of genes showing expression levels outside the ranges of the parents were also identified in the hybrids expressing heterosis. Further research is needed to determine if non-additive expression is associated with heterosis and to clarify the functional relationship between gene expression levels and heterosis for yield, or yield *per se*.

In addition to the Affymetrix arrays, a 16,000 feature array consisting of 70-mer oligonucleotides developed from *M. truncatula* sequence has been used to evaluate alfalfa for various traits (Deavours and Dixon 2005; Aziz et al. 2005; Chen et al. 2008). Because these arrays are based on longer oligos than Affymetrix arrays, a higher percentage of genes show a hybridization signal (Aziz et al. 2005). These experiments have been used to evaluate the effect of transgene insertions on global gene expression (Deavours and Dixon 2005), identify genes possibly associated with glandular trichome development (Aziz et al. 2005), and identify dehydration responsive genes (Chen et al. 2008).

Metabolite profiling is essentially in its infancy in alfalfa, with only limited research being done. Deavours and Dixon (2005) profiled metabolites of transgenic alfalfa and determined that additional secondary products were produced in the transgenic line compared to non-transgenic controls. Metabolite profiling will undoubtedly become more important as protocols for high-throughput analysis are further refined.

The value of gene expression profiling, metabolomics, and other advanced genomic technologies in terms of cultivar development in alfalfa is not clear. Obviously, these technologies offer research scientists an impressive ability to identify genes that may be involved in various traits, and these genes can become

"candidates" for QTL analyses. One obvious way to use genes identified from array experiments is to base genetic marker assays on these candidates using SNP, and hence assisting in targeting these genes to mapping studies for the relevant phenotype.

8.5 Genome Sequencing and Resequencing

Large-scale sequencing of alfalfa has not occurred yet, although several proposals are in the formative stages at the current time. The *M. truncatula* genome sequence is nearly complete (Young and Udvardi 2009), and will serve as the framework on which all alfalfa genomics work will be built in the future. Significant amounts of high-throughput sequencing will likely be done on alfalfa in the near future, enabling the detection of large numbers of SNPs, and the development of SNP arrays capable of assaying hundreds or thousands of genomic locations simultaneously.

8.6 Breeding of Transgenic Varieties

Transgenic cultivars of alfalfa are just beginning to be developed. Cultivars resistant to the herbicide glyphosate (Roundup®) were commercialized briefly in 2005 before being pulled from the market pending further environmental review. These cultivars were developed by incorporating two unlinked trangenes in order to develop synthetic cultivars in which nearly every plant has at least one copy of the gene (Samac and Temple 2004). A number of other traits are in the pipeline for eventual commercialization, including altered cell wall composition (Bouton, 2007). Breeding methods to incorporate transgenes into commercial cultivars were suggested by Woodfield and Brummer (2001). More information on the application of genetic engineering to trait development in alfalfa can be found in Chapter 4.

8.7 Interspecific Hybridization

In the genus *Medicago*, interspecific crossing was reviewed previously (McCoy and Bingham 1988; Quiros and Bauchan 1988). Hybrids between *M. sativa* and *M. arborea* were obtained, and their progenies displayed morphological traits typical of each parent; traits such as large seeds are being introgressed into alfalfa (Bingham 2005). Asymmetric *M. sativa* × *M. arborea* hybrids were also generated using cytoplasmic male sterile *M. sativa* as the female parent (Armour et al. 2008). Introgression of some of the *M. arborea* genome into *M. sativa* has been established, using morphological and DNA markers for anthracnose disease resistance.

Somatic hybridization can be obtained by *in vitro* protoplast fusion and regeneration from hybrid calli. This technique has not given practical results to date, but is still utilized to introgress traits from wild relatives. Hexaploid somatic hybrids

were obtained between *M. sativa* and *M. arborea* (Nenz et al. 1996); their morphology was generally intermediate between the parents, and they showed considerable genome rearrangements. Somatic hybrids of *M. sativa* with the annual species *M. rugosa* and *M. scutellata* were obtained by protoplast electrofusion (Mizukami et al. 2006). The number of chromosomes, variable in the hybrid callus, was reduced to that of *M. sativa* during proliferation and regeneration. Introgression of *M. rugosa* chromatin into *M. sativa* chromosomes was demonstrated by GISH. Hybrids involving *M. scutellata* were female sterile. Partial resistance to alfalfa weevil appears to have been transferred from *M. rugosa* to *M. sativa*.

9 Seed Production

Genetic, agronomic, and physiological analysis of seed production in alfalfa and its improvement through plant breeding are important because of the influence of this trait for the commercial development of a given variety and as a consequence for the dissemination of genetic progress achieved for other agronomic traits. The main world producers and exporters of alfalfa seeds are the USA (primarily in California and the northwestern states of Idaho and Washington), Canada, and France.

The analysis of the yield components showed the importance of architecture of seed crops and showed that the morphological characterization has to be considered to avoid the drifts that are likely to occur during seed multiplication generations.

9.1 Variation due to Genotype and Environment

Seed yield in a field is commonly analyzed through components related to number of pods per unit, number of seeds, and mean seed weight. It is necessary to analyze the genetic and physiological bases of seed yield and, in order to speed up the breeding process, to identify a newly defined trait which has to be heritable, easy to measure and highly correlated with seed yield.

In alfalfa, the variability among and within cultivars is very important for seed yield and all components (Bolaños-Aguilar et al. 2000). The environment greatly affects seed yield. With the common current techniques for seed production (low plant density, wide row spacing, clipping, the presence of wild pollinators), the soil and climate conditions which were favorable to a high accumulation of biomass are also favorable to high seed yield. The seed yield increase is achieved through a higher number of inflorescences per unit area and through a higher mean seed weight per inflorescence (Figure 11). These data were collected over many locations with two cultivars. The crops which are far above the general trend were those without clipping. However, in such conditions, major risks such as lodging, pod parasites, or lack of pollinators if the weather conditions are poor at flowering time, can result in crop failure. In a given sward, the mean number of inflorescences per

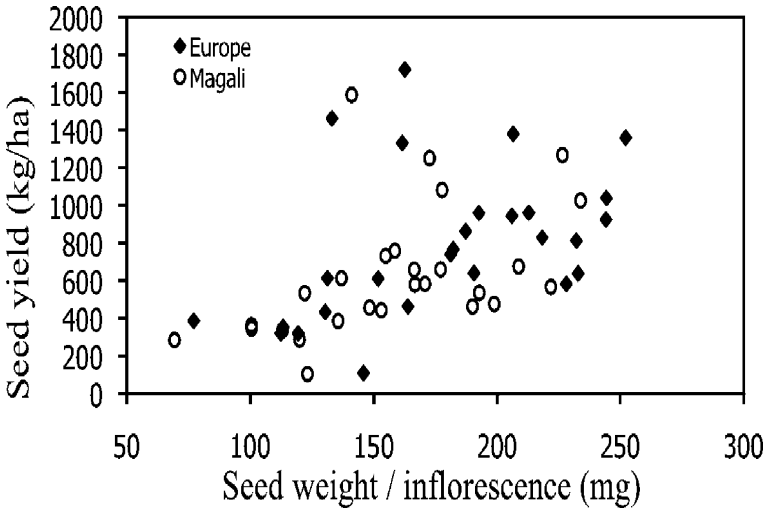

Fig. 11 Relationship between seed weight per inflorescence and seed yield for two varieties cultivated in a broad range of environmental conditions (from Bolaños-Aguilar, 2001)

stem is the same whatever the number of stems per plant. In the first year of production, most plants had one or two fertile stems, while in the third year of production, there were fewer plants and those with many stems contributed a significant part of the seed yield. Data also suggest that at the genotypic level, there is no negative relationship between vegetative biomass production and seed yield.

Genotype × environment interactions are highly significant for seed production but the ranking of cultivars tends to remain similar (Bolaños-Aguilar et al., 2002). As a consequence, the number of sites to evaluate cultivars may be low, but the sites with a high mean yield will be the most discriminating.

Broad sense heritability of seed yield components is high. This was found to be especially true for seed weight per inflorescence. The inheritance proved to be predominantly additive, making it possible to exploit the GCA value of genotypes when defining initial polycross parents for synthetic construction.

9.2 Consequences in Breeding and Production

Seed weight per inflorescence may be used as a selection criterion on individual spaced plants. A divergent selection was performed on this trait and proved to be very efficient (Huyghe, unpublished data). This trait could easily be included in the early steps of the breeding process. Indeed, the low genetic gains observed in seed yield are mainly explained by the fact that measuring seed yield on spaced plant is meaningless as it is mainly influenced by the number of stems or because seed yield is taken into account at a very late stage when it is possible to run seed yield trial.

Agronomic practices have been greatly improved over the last decades. They could be further modified in order to maximize seed yield production while avoiding the main agronomic failures due to a poor pollination and early lodging.

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Red Clover

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1 Introduction

Red clover (*Trifolium pratense* L.) is a perennial forage legume of limited persistence, mainly used for cutting in grass–clover leys of 2–4 years of duration, but also occurring naturally in permanent grassland. Among the forage legumes, in terms of seed produced and marketed worldwide, and in numbers of cultivars available, red clover ranks second after alfalfa (*Medicago sativa* L.) but still higher than white clover (*Trifolium repens* L.), although the latter has been gaining importance in the last decades.

Red clover can be grown in pure stand but is best mixed with tall growing grasses, such as Italian or hybrid ryegrass (*Lolium multiflorum* Lam. or *Lolium boucheanum* Kunth) under moist and winter mild conditions, timothy (*Phleum pratense* L.) or meadow fescue (*Festuca pratensis* Huds.) under harsher conditions or tall fescue (*Festuca arundinacea* Schreb.) under dryer conditions. Admixture of a grass increases yield and yield stability, prevents from nitrate leaching and enhances nitrogen fixation efficiency (Nyfeler et al. 2009). Red clover can also be used as a starter legume to facilitate establishment of white clover in complex mixtures for long-term grassland mixtures (Suter et al. 2008b)

Seedlings of red clover form a purely vegetative primary shoot, with each leaf carrying an axillary bud capable of forming lateral shoots which have the potential of becoming generative stems. Stems have a limited number of extended internodes (most often 4–6) and terminate with a flower head, while short side branches capable of sub-branching can develop from lower leaf axils. After cutting, new shoots form near the plant basis which turns into a "crown" as the plant is aging. During the usual lifetime of a red clover stand, the root system is dominated by a strong tap root developing from the primary seedling root. Root depth is intermediate between that of shallow-rooting white clover and that of deep-rooting alfalfa, reaching as deep as 1 m. Eventually, adventitious roots form at the crown, rarely at the lowest

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axils of prostrate lateral shoots. These features make the persistence of red clover stands much dependent on the sustainable performance of established individuals.

Julen (1959) describes in detail red clover breeding and research activity in the first half of the 20th century. The unique monograph of Taylor and Quesenberry (1996) gives a comprehensive overview of all aspects of red clover science, from history, systematics, botany and pathology through breeding to agronomy and seed production. In this chapter, we will summarise those aspects most relevant to breeding and focus on recent knowledge with particular attention to molecular techniques.

2 Origin and Systematics

Red clover is thought to originate from southeastern Eurasia, near the Mediterranean, and is indigenous to Europe, the Near East, North Africa and central Asia, while it has been introduced deliberately for use as forage in the rest of the world. Red clover is one of the forage plants with the longest cultivation history, probably second only to alfalfa. The first mention of clover in an agricultural setting is by Albertus Magnus (1193–1280) in "De Vegetabilibus" and it is likely that red clover was meant (Jessen 1867). The replacement of fallow by clover in the medieval three-field economy boosted what may be called a first green revolution. In 1566, Dodoens described clover cultivation as an established practice in Brabant and observed that red clover cultivated on arable fields "grows much more vigorously and taller than the red clover on the meadows" (Freudenthaler et al. 1998). It is likely that the seed for cultivating red clover in Europe took a route from the Near East to Spain (then ruled by the Moorish), and from there by sea to the Netherlands and Brabant, from where it spread to the rest of Europe (Zeven and de Wet 1982). Recent molecular evidence (Herrmann et al. 2005) showed a clear separation between local landraces and spontaneous populations of red clover from permanent meadows in Switzerland, while the group of landraces was difficult to separate from traditional and advanced cultivars of any European origin. This suggests that spontaneous red clover developed by natural spreading, independently from the assumed route of introduction of cultivated forms, and that development and propagation of the latter was little influenced by gene flow from indigenous populations.

According to the classification of Zohary and Heller (1984), red clover is placed in section *Trifolium* Zoh. and is selected as the type species (lectotype) of the genus *Trifolium* L. Taxonomic relationship with other species of the genus are discussed by Taylor and Quesenberry (1996). Different proposals were made to subdivide *T. pratense* L. taxonomically. Stebler and Volkart (1913) distinguished between *T. pratense* var. *sativum* Schreb. for the cultivated and *T. pratense* var. *spontaneum* Willk. for the wild (spontaneous) forms. Julen (1959) subdivided the cultivated forms into European *T. pratense* var. *subnudum* Witte and American *T. pratense* var. *expansum* Haussk. Hess et al. (1970) use a similar subdivision and treat *T. pratense* as a group of species, containing *T. pratense* L. (sensu stricto) for the spontaneous forms, *T. sativum* Crone for European and *T. expansum* Waldstet. et Kit.

for American cultivated forms, as well as white flowering *Trifolium nivale* prevalent at alpine altitude. None of these suggestions has been adopted in a general way, and *T. pratense* L. is the lowest, generally accepted taxonomic unit of red clover.

3 Varietal Groups

North American red clover is distinguished from European red clover by strong pubescence which is thought to help prevent leafhopper damage (Pieters and Hollowell 1937). Generally, European germplasm is poorly adapted to North American conditions and vice versa. Two main groups of European cultivars can be distinguished according to flowering time. Early red clover is adapted to southern latitudes and is characterised by rapid regrowth capable of re-flowering, while the late red clover of the northern latitudes usually remains completely vegetative after the first, generative cut. Julen (1959) assigned early red clover to a subvar. *praecox* and late red clover to a subvar. *serotinum*. A similar distinction was made in North America where early-maturing types were designated medium or double-cut, while the late-maturing types were called mammoth or single-cut (Smith et al. 1985). While modern plant breeding has let the borderline between these groups become less sharp so a taxonomic distinction is no longer justified, North American and European, as well as early and late cultivars, remain predominantly adapted to their regions of origin and use as outlined above.

The OECD list of cultivars (OECD 2009) and important national or recommended lists distinguish between diploid ($2n = 2x = 14$) and tetraploid ($2n = 4x = 28$) cultivars. The VCU requirements for listing of a new cultivar are usually different for the two ploidy levels. In Switzerland, short-lived cultivars of a maximum duration of 2 years are listed as "Ackerklee" (field clover), while more perennial cultivars are listed as "Mattenklee" (meadow clover), and for listing, VCU requirements must be met according to ploidy level for the respective groups (Suter et al. 2008a). The German descriptive list of red clover cultivars (Bundessortenamt 2007) has a category of cultivars reserved for amenity purposes, these have been derived from spontaneous red clover ("Wiesenrotklee").

4 Genetic Resources and Utilisation

In all regions of red clover cultivation, locally adapted populations have historically developed from the practice of re-sowing seed harvested within a restricted area around the source field, often limited to one particular farm itself. In this way, landraces were created which were named for the farmer or region where they originated. Farmers were well aware that their home-grown seed had specific adaptation to their growing conditions and were careful not to spoil this by introducing seed of unknown foreign provenience. Red clover farm landraces were apparently maintained with particular care because red clover seed of high quality was long difficult

to obtain on the market. Thus, the landraces may be regarded as the result of a semi-conscious selection by the farmer-grower.

Until about 1950, a great diversity of such landraces was cultivated in many regions of the world. This diversity often served as starting point for emerging systematic breeding programmes. Around 1970, a strong decline in the use of red clover landraces was observed, associated with the greater ease in obtaining seed of high-quality cultivars (Taylor et al. 1977; Boller 2000). Consequently, the tradition of maintaining farmer landraces was quickly abandoned. Luckily, an important part of this diversity has been preserved by collection and conservation in genebanks. Locally adapted, cultivated germplasm may still be found in traditional farming systems of less developed countries.

Landraces play a more prominent role for red clover breeding than in other forage species. This is due to the long cultivation history of the species and to the relatively strong differentiation between cultivated and wild (spontaneous) forms. The use of wild forms of red clover in practical breeding is mostly restricted to introducing particular characteristics and requires several cycles of backcrossing before agronomic performance approaches that of advanced cultivars.

Two searchable germplasm collections are most important for red clover accessions maintained ex situ: The ECPGR *T. pratense* Database (www.ecpgr.cgiar.org/databases/Crops/trif_pra.htm) maintained by the Institute of Agrobotany, Tápiószele, Hungary, actually (April 2009) lists 2,294 accessions held by 19 genebanks or other institutions of 15 European countries, of which 43% are commercial varieties or breeder's lines, 21% landraces, 25% wild or semi-natural ecotypes and 6% material of unknown type. The USDA National Plant Germplasm System, United States (www.ars-grin.gov/npgs/) lists 1,038 accessions, with 35% cultivars and other selected material, 16% landraces and 22% wild ecotypes. Eighty-five accessions are defined as a core subset. This core collection was established based on research by Kouamé and Quesenberry (1993) and was characterised for genetic diversity based on isozymes (Mosjidis and Klingler 2006) as well as morphological traits and SSR markers (Dias et al. 2008).

5 Breeding Objectives

5.1 Forage Yield and Persistence

Red clover is a high-yielding forage legume suitable for cutting to produce fresh or conserved fodder but does not tolerate intensive grazing. A high and reliable dry matter yield, resulting from up to four cuts per full harvest year, is therefore a key breeding objective. However, realised yield potential of red clover is most often limited by insufficient persistence, which in turn is affected by various diseases. Therefore, breeding red clover for yield per se is rarely carried out without simultaneously paying attention to resistance against biotic and abiotic stresses affecting persistence.

Key fungal pathogens threatening the desired survival of red clover depend on the target region of cultivation. In cooler areas with strong winters and long snow cover, *Sclerotinia trifoliorum* Eriks. causes crown rot, a major disease deserving attention in breeding programs. Techniques to improve *Sclerotinia* resistance by inoculation with mycelium suspensions were developed by Frandsen (1946) and refined by Dixon and Doodson (1974). Marum et al. (1994) suggested the use of ascospores for more predictable inoculation. These various techniques are widely used by breeders, and *Sclerotinia* resistance is systematically assessed in official variety testing. In warmer climates, southern anthracnose, caused by *Colletotrichum trifolii* Bain & Essary, can lead to loss of individual plants and significant sward damage during summer. In the southern part of the clover belt of the United States, the disease has been controlled by the use of resistant cultivars already in the 1950s (Taylor 2008). However, as these regions are less important for clover cultivation, breeders have paid more attention to northern anthracnose and its causal agent, *Kabatiella caulivora* (Kirchn.) Karak. (Taylor et al. 1990). More recently, *C. trifolii* appeared to benefit from rising temperatures in many areas, and by causing leaf and stem symptoms (Figure 1) but more importantly crown decay, the disease has become a limiting factor for yield and persistence in previously less affected regions (Boller et al. 1998). Consequently, inoculation techniques were developed and applied to improve resistance (Schubiger et al. 2003).

Fusarium spp. are often encountered in association with root rot but their role as pathogens is not quite clear. For example, an increased abundance of *Fusarium* spp. is observed as a side-effect of root breakdown due to various other stresses such as summer drought or southern anthracnose. However, some breeding efforts are

Fig. 1 Leaf symptoms of southern anthracnose (*C. trifolii*) on red clover. Typically, leaf petiole and blade turn completely black above a clearly separated symptomless zone of the petiole. Shorter zones of black necrosis may appear further down the petiole and result in breaking (Photo F. Schubiger)

devoted to resistance against Fusarium with the aim of improving persistence. Rufelt (1985) developed a dipping technique to inoculate roots with conidia of Fusarium spp. but progress in resistance using recurrent selection based on this technique was very slow (Venuto et al. 1999). Nedelnik (1992) concluded that mortality of red clover plants in the field was caused by a complex of biotic and abiotic factors and was not related to *Fusarium* resistance observed in greenhouse conditions.

Red clover persistence is also negatively affected by insect and nematode pests. Although root-parasitising insects like the root borer (*Hylastinus obscurus* Marsham) and the clover root curculio (*Sitona hispidula* F.) have been advocated as being involved strongly in lowering stand persistence of red clover (Leath and Byers 1973), no targeted selection for resistance has been carried out. In contrast, nematode resistance is a major concern in red clover breeding. In temperate Europe, the stem and bulb eelworm *Ditylenchus dipsaci* (Kühn) Filipjev often affects red clover grown in narrow crop rotations and therefore, systematic selection for nematode resistance including artificial inoculation has been carried out almost since the establishment of modern breeding programmes. Related techniques were first described by Akerberg et al. (1947) and evaluated in detail by Bingefors (1951). These techniques are used systematically by red clover breeders to improve persistence. In the USA, successful selection for resistance against root-knot nematodes (*Meloidogyne* spp.) has been carried out (Quesenberry et al. 1989).

5.2 Resistance to Foliar Diseases

Among the various foliar diseases occurring on red clover, powdery mildew (causal agent *Erysiphe polygoni* DC.) is getting the most attention in plant improvement programs. This may be due to its prominent appearance, often completely covering the foliage with its white mycelium in late summer. However, it usually does not greatly affect yield and disappears without weakening regrowth of the plants after cutting. Nevertheless, resistance to powdery mildew is assessed and taken into account in official cultivar testing and progress in resistance is sought by plant breeders. Greenhouse conditions favour the disease and offer easy opportunities for screening. *Stemphylium sarcinaeforme* (Cav.) Wiltshire occurs widespread, it causes target spots and can decrease yield of affected growth cycles quite considerably. Important differences in cultivar susceptibility have been observed (Berg and Leath 1996).

5.3 Quality Characters

Red clover is rich in protein, but contains little soluble carbohydrates and tends to be less digestible than forage grasses. However, this drawback is easily coped with by growing red clover in mixed stands with highly digestible grasses like Italian ryegrass. Red clover is an ideal complement to such grasses because of its high protein

content. Therefore, nutritive value in general and digestibility in particular is a less important breeding objective than with grasses, and current attempts to improve forage quality of red clover largely focus on secondary plant metabolites. Red clover contains high concentrations of estrogenic compounds which are the basis of medications against hormonal disorders with women (Coon et al. 2007). However, when fed to ewes before mating, it can hinder conception severely. Breeding red clover for a low content of formononetin is the objective of specific breeding programmes which have resulted in the release of cultivars with reduced contents, both in Europe and in New Zealand (Boller 1994; Rumball et al. 2005). More recently, interest has been raised for genetic variability in activity of the enzyme polyphenol oxidase (PPO) contained in red clover (Lee et al. 2004). PPO is made responsible for the slower breakdown of red clover protein during ensiling when compared to alfalfa (Sullivan and Hatfield 2006). Red clover with high activity of PPO might contribute to limit nitrogen losses from ruminant husbandry systems.

5.4 Seed Yield

Seed yield is a very important character for the market success of red clover cultivars. Intensive selection for as forage characteristics seems to have hindered progress in seed yield and some cultivars with excellent agronomic features in terms of forage production are of little market importance because of their limited seed yield. This is particularly true of tetraploid cultivars which yield 20–50% less seed than diploid ones. Selection for seed yield is often carried out only at a late stage of the breeding process.

Seed production potential of red clover has been found to be negatively correlated with agronomic traits such forage production potential (Steiner et al. 1997). However, this is not a general phenomenon. In a mapping population, Herrmann et al. (2008) found both persistence and seed yield to be positively correlated with length of stem, and QTL for these three traits were located in the same genomic region. However, seed yield components were influenced by no less than 38 QTL, underlying the complex nature of these traits (Herrmann et al. 2006). Prolific flowering was positively correlated with seed yield per plant, providing a useful indirect selection criterion for seed yield.

5.5 Symbiotic Performance

Red clover lives in symbiosis with *Rhizobium leguminosarum* biovar *trifolii* and is capable of providing nearly 400 kg of fixed nitrogen per ha per year (Taylor and Quesenberry 1996). Direct selection for nitrogen fixing efficiency is complicated by specificity of the host–symbiont relationship (Nutman 1984). Consequently, few efforts have been undertaken to improve symbiotic performance through breeding. However, using ^{15}N methodology, genetic variation in the percentage of N

derived from the atmosphere ($\%N_{dfa}$) among full-sib families of red clover was found to be positively correlated with dry matter yield (Boller and Nösberger 1994), and the differences were consistent across a range of mineral N availability. Therefore, selecting for high dry matter yield at a given low level of nitrogen availability should be efficient in improving symbiotic performance at any level of soil N availability.

6 Breeding Achievements

Red clover was one of the first forage plants dealt with at the dawn of modern plant breeding around the turn from the 19th to the 20th century. For example, the impact of self-incompatibility on breeding techniques was discovered in red clover by one of the pioneers of a science-based plant breeding (Martinet 1903). The early importance of red clover in plant breeding is related to its prominent role in the historical development of farming systems in Europe, recognising the potential of red clover to contribute to soil fertility long before the process of symbiotic nitrogen fixation had been understood. However, the availability of highly performing landraces made it difficult for early red clover breeders to obtain a competitive product. Julen (1959) stated that most red clover cultivars in agricultural use were those landraces which had proven best performance in cultivation testing. Both public and private institutions became more active in systematic red clover breeding after World War II, and the OECD list of cultivars eligible for certification of 1968 already contained 110 cultivars of red clover. At that time, this was the highest number for a single forage species. The current OECD list of cultivars eligible for seed certification (OECD 2009) contains 255 cultivars of red clover, of which 45 were added in the past 5 years, while 24 had disappeared from the list of 2004.

Considerable improvement of persistence and hence yielding capacity over several years has been obtained by breeding programmes in different parts of the world. The most prominent example is the development of highly persistent red clover cultivars at the Wisconsin Agricultural Experiment Station, resulting in an improvement of reliable stand duration from an initial two to four seasons after four decades of dedicated selection (Smith 2001). Similarly in Japan, targeted selection for over 20 years, based on a combination of maternal line and individual plant selection resulted in cultivars with considerably improved persistence (Isobe et al. 2002). Great progress in persistence of early flowering, multiple-cut types of red clover was obtained by the use of Swiss Mattenklee (Boller 2000). This genetic resource, originating from local cultivars of cultivated clover in some regions of Switzerland, has been successfully used to create cultivars of outstanding longevity under temperate European conditions. This programme is also a good example for the success of plant breeding in coping with new diseases. Resistance to southern anthracnose, which was clearly insufficient in existing Mattenklee cultivars, was markedly improved and new cultivars of this type are again as persistent as the old ones were before the disease became so prominent (Boller et al. 2004).

No studies on genetic gain in yielding potential of red clover have been published. Recent reviews of breeding progress in Europe (Abberton and Marshall 2005) and the United States (Taylor 2008) failed to identify appropriate references. However, the long lifetime of red clover cultivars supports the observation that breeding progress in red clover, in terms of forage yield potential, is rather slow. For example, the cultivar Mt. Calme, developed in the 1920s from an old landrace (Boller 2000) and tested successfully in independent, official tests for the first time between 1928 and 1932 (Neuweiler 1932), still persists on the very restrictive list of recommended cultivars for Switzerland and outyields newer recommended cultivars of short-lived field clover (Suter et al. 2008a). Similarly, the cultivar Gumpensteiner, also an improved landrace, first released shortly after World War II and gradually improved until the early 1970s, is still the most used and best recommended red clover cultivar in Austria (Krautzer 2003). In the United States, the cultivar Arlington, registered in 1973 (Smith et al. 1973), still serves as check variety in Wisconsin and usually yields just marginally less than new releases in the first 2 years of stand (e.g. Smith 2001). Of the 129 red clover cultivars listed in the OECD list of 1981, 56 are still to be found on the edition of 2009 (OECD 2009).

Substantial improvement in forage yield of red clover was obtained by inducing polyploidy. The first successful induction of polyploidy in red clover was reported during World War II (Levan 1940), and reproducible techniques became available in the 1950s (Brewbaker 1952). Compared to their diploid ancestors, tetraploid cultivars yield significantly more forage dry matter. Disease resistance and persistence are also improved (Boller et al. 2003). However, seed yield of tetraploids is markedly lower. The relative market success of tetraploid red clover greatly depends on the possibility to reliably produce seed and on the willingness of farmers to reward the agronomic quality of tetraploids with a higher seed price.

7 Breeding Methods and Specific Techniques

Red clover is an allogamous species with a strong gametophytic self-incompatibility system. Although pseudo-self-compatibility occurs occasionally and can be exploited by high-temperature treatment, it is of no relevance in the presence of compatible pollen and can be neglected when inter-crossing individuals. Selected individuals can be intermated by allowing open pollination by naturally occurring bee pollinators with adequate spatial isolation or by using bee cages. Bumblebees (*Bombus* spp.) are more efficient at pollinating red clover than honeybees (*Apis mellifera* L.). This is particularly true of tetraploid red clover which can only be efficiently pollinated by certain *Bombus* spp. with long nozzles, such as *Bombus pascuorum* Scopoli. Conversely, *Bombus terrestris* L. bumblebees are unable to pollinate tetraploid red clover due to its longer corolla, which they often puncture to rob nectar without getting into contact with anthers or stigma. If only a small number of progeny per individual is required, manual pollinations can be made with

Fig. 2 Hand pollinating red clover; from *left* to *right*: removing wings and keels of female parent with forceps; collecting pollen by "tripping" flowers of male parent with folded cardboard piece; applying pollen to stigma (Photo G. Brändle)

isolated flower heads. The use of detached stems placed in tap water shortly before flowering and maintained at about 20/15°C day/night temperatures until seed ripening is possible. Before applying pollinator plant pollen, individual flowers of the plant selected as the female parent are best prepared by removing wings and keel with forceps (Figure 2). Apart from making the stigma more easily accessible, this releases the plant's own pollen which will not dilute that applied with a pollinating instrument, such as a toothpick or a folded cardboard piece. Seed set of about 75% for diploids and about 50% for tetraploids can be expected.

Recurrent mass and maternal line selection are the most frequently used breeding methods to develop the breeding population. Candidate varieties are usually created by combining superior progenies of a number of elite parent individuals. The progenies may be obtained by open pollination or pair crosses. Progenies are tested in small plots or rows, and the best progenies are allowed to intermate to form the new candidate variety. The resulting cultivar may be called a synthetic with a fixed number of half- or full-sib families as components. Classical polycross breeding involving vegetative maintenance of parent individuals during progeny testing is rarely carried out because vegetative propagation of red clover is difficult and long-term maintenance of clones is risky.

Tetraploids are most often obtained by colchicine treatment of young seedlings. Recommended concentrations vary between 0.02 and 0.4%. In the authors' breeding programme, a small piece of cotton is immersed in a 0.1% colchicine solution and wrapped around the apical meristems of seedlings with just unfolded cotyledons. Non-responsive seedlings are removed after 4 weeks. About 10% of the responsive plantlets survive and are allowed to flower. Pollen is inspected after treatment with sulphuric acid (Funke 1956) to identify tetraploid flower heads (Figure 3). Pairs of tetraploid plants are then manually intercrossed. To found a new basic population, at least 50 crosses involving at least 30 nonrelated individuals of the colchicine-treated generation (C0) are carried out. The material is advanced for at least two generations of mass selection before elite individuals are selected as parents of progenies for variety synthesis.

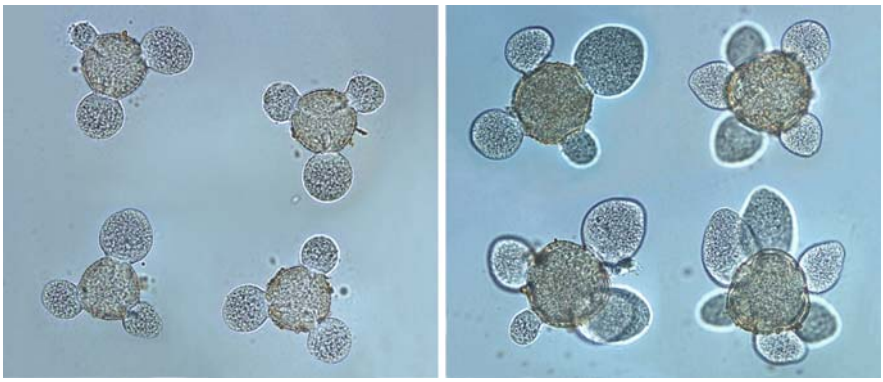

Fig. 3 Microscopic view of pollen of diploid (*left*) and tetraploid (*right*) red clover after treatment with Funke's sulphuric acid. Pollen stroma is profusing through pollen tube germination pores. Pollen of diploid plants is tetraeder shaped with three pores visible in one plane. Pollen of tetraploid plants is octaeder shaped with either four pores visible in one plane or six (two times three) pores in two planes (Photo F. Schubiger)

As an alternative to colchicine treatment, nitrous oxide has been proposed (Berthaut 1968), and sexual polyploidisation using unreduced gametes is also possible (Parrott and Smith 1986; Simioni et al. 2006). However, these methods have not gained widespread importance in practical breeding.

8 Development and Application of Molecular Genetic Tools

The combination of conventional approaches with molecular genetic tools has been shown to improve efficiency and precision of plant breeding in a variety of plant species (Collard and Mackill 2008). In red clover, progress in improvement of yield potential has been limited in the past, probably due to the multitude of stress factors breeders have to account for which prevents a more targeted selection for yield. Consequently, most conventional breeding efforts are invested into maintenance of persistence in response to evolving pathogen populations or changing abiotic pressures. Molecular markers which allow securely identifying individuals homozygous for important resistance genes would greatly facilitate subsequent phenotypic selection for yield-related, polygenic factors. In addition, markers for important traits conferring longevity per se would allow to minimise the particularly time-consuming phenotypic selection for this characteristic. Finally, molecular markers may also assist breeders in the targeted characterisation and utilisation of genetic resources for broadening the breeding gene pool and for optimal selection of parental combinations in complex breeding schemes.

Despite the numerous promising areas of application, examples for the actual application of marker-assisted selection are scarce for forage and turf species and for red clover no such attempts have been reported so far. However, in recent years

numerous efforts have produced a substantial array of molecular genetic tools which have so far been predominantly applied for the characterisation of genetic diversity and the investigation of the genetic control of target traits such as persistence or seed yield.

8.1 Molecular Marker Development and Genome Sequencing

As in most forage and turf species, the lack of sequence-specific markers has favoured the employment of anonymous markers such as RAPD, AFLP or ISSR (see Chapter 4 for details). As late as in 2003 the first larger set of sequence-specific markers was developed for red clover (Isobe et al. 2003). Based on a genomic cDNA library from red clover seedlings, the authors developed 248 restriction fragment length polymorphism (RFLP) markers. Although these markers were successfully used to construct the first linkage map in red clover, widespread application was hampered by the labour generally required to detect RFLP markers. More recently, a vast resource of more than 1,300 genomic and gene-associated simple sequence repeat (SSR) markers has been made available (Kölliker et al. 2006; Sato et al. 2005). These markers showed excellent transferability across different red clover populations and detected between 2 and 25 alleles per locus.

Although the complete genome sequence of red clover is not available up to date, with the sequencing of 26,356 expressed sequence tags (ESTs), an extensive resource for genomic research has been created (Sato et al. 2005). The ESTs corresponded to 9,339 unique genomic sequences, of which 78% showed sequence similarity to known genes, mainly of *Arabidopsis* and rice.

8.2 Linkage Mapping and QTL Analysis

The first genetic linkage map of red clover, one of the most fundamental prerequisites for molecular breeding approaches, was developed by Isobe et al. (2003) and consisted of 157 RFLP loci with an average distance between two loci of 3.4 cM. Later, two additional linkage maps were produced based on 1,286 SSR and 148 RFLP markers (Sato et al. 2005) and based on 216 AFLP and 42 SSR markers (Herrmann et al. 2006). In an attempt to provide a more comprehensive resource for further investigations, Isobe et al. (2009) produced a red clover consensus linkage map which is the first such map for an outbreeding forage species. It is based on six mapping populations, including the populations of the previously mentioned studies, and consists of 1,804 marker loci. The map has a total length of 836.6 cM and the average distance between two loci is 0.46 cM. Comparisons of the maps of individual parents with the consensus map showed a largely conserved marker order across all seven linkage groups. Thus, the map presents an invaluable resource for use as a reference for the genetic analysis in any other red clover germplasm. In the same study, a low level of linkage disequilibrium (LD) and no correlation between

LD and genetic distance was observed. This confirms the high level of out-crossing generally observed in red clover and at the same time highlights the difficulty of using association mapping as an alternative to QTL analysis in this species. If LD decays within 100 kb in a red clover mapping population, more than 4,400 markers would be required for genome-wide LD mapping, which is not feasible with the resources currently available.

Despite the availability of molecular tools and the economic importance of the species, so far only two studies investigated the association of molecular markers with phenotypic characteristics in order to provide means for marker-assisted improvement of target traits (Herrmann et al. 2006; Herrmann et al. 2008). Both studies were based on the same mapping population produced by reciprocal crossing of one genotype of the field clover cultivar "Violetta" and the Mattenklee cultivar "Corvus". For seed yield per plant, a trait difficult to improve through phenotypic selection, three QTL were identified on three different linkage groups which together explained 33.8% of the phenotypic variation observed (Herrmann et al. 2006). For persistence, a number of different characteristics were evaluated. A weighted average of vigour scores assessed during two winters and three growing seasons was identified as the optimal method to phenotype persistence and one significant QTL explaining 12.2% of the total phenotypic variation was identified (Herrmann et al. 2008). The markers linked to these QTL provide a first basis for future marker-assisted efforts for the improvement of these two important traits.

8.3 Characterisation of Genetic Diversity

As an obligate outbreeding species, red clover is characterised by high intra-species diversity. Unusually high levels of genetic variation were observed in restriction digests of red clover chloroplast DNA (Milligan 1991). This suggests that not only the nuclear genome but also the plastid genome of this species shows high levels of diversity at the population level.

Molecular markers may support red clover breeders in their decisions on how to maintain or increase genetic diversity in their germplasm collections. For example, Ulloa et al. (2003) showed in a comparison of 12 Chilean advanced breeding populations with eight cultivars from Chile, Argentina, Uruguay and Switzerland that the breeding populations together with the Chilean and Argentinean cultivars formed a distinct group while the Uruguayan and the Swiss cultivars were clearly separated. The authors conclude from their survey that the genetic diversity within the Chilean breeding germplasm may be limited and suggested to broaden the gene pool by including genetically more divergent parents. In an extensive survey of 120 red clover populations, genetic diversity was found to be particularly high in Swiss wild clover populations and Swiss Mattenklee landraces (Herrmann et al. 2005). Thus, these accessions may be particularly valuable as genetic resources for the further improvement of red clover cultivars as well as for conservation and restoration of biodiversity.

Although molecular markers can certainly assist plant breeders in their efforts to exploit genetic diversity, estimates of genetic diversity based on anonymous genetic markers often poorly correlate to the diversity of phenotypic traits (Mosjidis et al. 2004; Dias et al. 2008). Therefore, in the future the development of a novel generation of molecular markers which are directly linked to functional characteristics is of outmost importance to enable efficient utilisation of genetic diversity.

9 Seed Production

In the past, seed production of red clover often was a by-product of forage production and was carried out on old fields previously used for forage. Nowadays, most red clover seed is produced far away from the region of use for forage in some specialised areas such as the Pacific Northwest of the United States. Loss of genetic stability during seed multiplication due to shift must therefore be prevented by limiting the number of generations between breeders' and certified seed. It is the breeder's responsibility to fix the number of generations allowed for seed increase. Total number of generations should generally not exceed four, of which only two should be allowed clearly outside the environment the cultivar had been selected in. At the usual seeding rate of 10 kg/ha and a seed yield expectation of 400–600 kg/ha (with large environmental variation), seed multiplication factors are clearly lower than in most grasses. A second seed harvest year obviously doubles this factor and this is another incentive to select for persistence.

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White Clover

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1 Introduction

White clover (*Trifolium repens* L.) is the most widely grown temperate forage legume and the most common forage legume in pastures grazed by sheep or cattle. A detailed overview of all aspects of white clover was given by Baker and Williams (1987). The agronomy of this species and its performance in mixtures with grasses has been previously reviewed (Frame and Newbould 1986, Frame et al. 1998, Laidlaw and Teuber 2001). Recent reviews outline the objectives of white clover breeding programmes in both the rain fed and irrigated regions of Australia (Lane et al. 1997, Jahufer et al. 2002), in the USA (Taylor 2008) and in New Zealand (Williams et al. 2007). In the UK, breeding of white clover was discussed by Rhodes and Ortega (1996). Williams (1987) gives a general review of white clover breeding and Woodfield and Caradus (1994) described progress over 60 years of plant breeding. The focus of this chapter is on developments since then, in particular highlighting breeding targets, especially increased resource use efficiency and coping with climate change, and recent progress in the application of molecular genetic knowledge to white clover improvement (Abberton and Marshall 2005).

2 Origin and Systematics

The genus *Trifolium* includes more than 250 species of which ten are of considerable agricultural importance (Zohary and Heller 1984). White clover is in section Lotoidea along with the other perennials *Trifolium Hybridum* (alsike clover) and *Trifolium ambiguum* (kura clover or Caucasian clover). Centres of diversity for clovers occur in the eastern Mediterranean, East Africa and South America (Zohary and Heller 1984). Recent work supports the Mediterranean origin of the genus in the Early Miocene period (Ellison et al. 2006). This work also provides evidence for *Trifolium pallescens* and *Trifolium occidentale* as the likely diploid progenitors

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of the allotetraploid white clover. The taxonomy and biosystematics of white clover were reviewed in Williams (1987) and a molecular phylogeny of the genus was presented by Ellison et al. (2006). The characteristic feature of white clover is its stoloniferous habit, i.e. it spreads by means of stolons or horizontal stems and thus has many active growing points. Much of the persistence of the plant, and its tolerance of defoliation and other stresses, are bound up with the effectiveness with which this stolon network functions as a storage reserve, means of growth and anchorage to the soil surface. The survival of a good network of stolons and the maintenance of the carbohydrate reserves stored in them are crucial if the clover is to compete effectively with its grass companion in early spring.

3 Varietal Groups

White clover varieties are characterised by their leaf size and fall into four groups (small, medium, large and very large). Leaf size is closely related to the size of the stolons and dictates the livestock system for which the varieties are best suited. Small leaf varieties are considered suitable for continuous hard sheep grazing, medium leaf types used under rotational grazing and large or very large leaf size cultivars mainly for lax cattle grazing or conservation. Caradus and Woodfield (1997) provided an overview of varieties in commerce. More recent statistics show that as of 1st January 2009 there are 186 varieties on the OECD list of white clover varieties (www.oecd.org) across the range of leaf size categories.

4 Genetic Resources and Utilisation

White clover breeding programmes use genetic resources from a range of sources to introduce variation. Traditionally novel plant material has been obtained by plant breeders through plant collection expeditions to geographic areas where germplasm with desired traits may be found. A good example is the improvement in early spring growth introduced into the Aberystwyth white clover breeding programme by using accessions collected in Switzerland (Rhodes and Ortega 1996). Plant breeders also use genebank collections as a source of genetic material. The European Central white clover Database (www.ecpgr.cgiar.org) contains passport data on 1350 accessions of white clover stored in 14 European genebanks as well as a portal for access to other genebanks and collections outside Europe. Description of these accessions is still rather limited and is mainly restricted to location with no molecular characterisation.

5 Breeding Targets

White clover is an outbreeding species with individual plants generally self-sterile. It is an allotetraploid or amphidiploid ($2n = 4x = 32$) that forms bivalents and

shows disomic inheritance. The breeding of white clover is strongly influenced by the range of biotic interactions it is subjected to and its near universal utilisation in mixed swards, most commonly with perennial ryegrass (*Lolium perenne* L.). Varieties bred in one country may not always perform well in another. Unlike many crops, maximisation of yield per se is not the main objective but rather the aim is to produce a balanced sward with a reliable, consistent white clover contribution. White clover/perennial ryegrass swards may be used profitably for many years and the reliability over time of the white clover contribution is a key concern of the farmer and, therefore, the breeder. The optimum level of this contribution may vary somewhat according to the management system, environment and the prime requirement from white clover. Thus, under rotational grazing by sheep or cattle in the UK a contribution of 30% as an average across the growing year may be considered satisfactory whereas in other circumstances this may be considered either too low or too high depending on whether N fixation or high intake and quality forage is the main objective.

5.1 Yield and Persistency

The development of a strong network of stolons is a pre-requisite of persistence and stolon characters have been a major focus of breeding efforts (e.g. Caradus and Chapman 1996, Collins et al. 1997). In the development of new white clover varieties, the likely on-farm use is an important determinant of the management regime used in selection and to some extent also the objectives of the programme. This in turn is strongly dependent on leaf size. Productivity is greater with larger leaf size but there is often a negative correlation between this and persistency. Thus, for instance, large-leaved Ladino types of white clover are usually considered as less persistent, although considerable variation for a range of traits exists within these forms (Annicchiarico 1993). The breaking of this negative correlation has been a major goal of breeders and has been achieved in some notable varieties (Woodfield and Caradus 1994). The effect of grazing management on persistence is of considerable importance in white clover and numerous studies have been carried out describing differences in performance between varieties, ecotypes or breeding lines (e.g. Brock and Hay 1996). Swift et al. (1992) report one of many studies comparing white clover performance under cutting and grazing regimes. Persistence has also been routinely selected for directly (e.g. Evans et al. 1996). Progress in the development of more persistent varieties has had major impacts on the reliability of white clover and this has been paralleled by an expansion of its potential. Long-term trials have shown that many modern varieties do not show marked reductions of clover content in mixed swards over 10 years or more irrespective of the level of applied N (Williams et al. 2003). Williams et al. (2000) showed that these varieties are capable of contributing significantly to highly productive systems with relatively low outputs and not just low-input/low-output systems.

5.2 Tolerance of Abiotic Stress

In general, breeding of white clover has focused on the improvement of a range of factors which together lead to increased persistence and reliability of clover content from year to year in a mixed sward. Lack of winter hardiness can contribute to poor performance and this encompasses the direct effects of cold temperature, snow cover and dehydration particularly due to desiccating winds. This has been a major focus of white clover breeding programmes in the UK and improvement in Scandinavia (Helgadottir et al. 2008). White clover breeding in New Zealand for European markets has also emphasised this trait (Caradus and Christie 1998). In white clover, the survival of a good network of stolons and the maintenance of the carbohydrate reserves stored in them are crucial if the clover is to compete effectively with its grass companion in early spring. Selection for cold tolerance has taken an in situ approach in which survivors from populations subjected to reliable cold stress in the field have been used in crossing programmes or has utilised artificial selection in freezing tanks or a combination of both (Collins et al. 2002). Biochemical characterisation of metabolic components associated with cold tolerance has been carried out for white clover and other forage species. Several have been implicated in improved winter hardiness, e.g. vegetative storage proteins (Goulas et al. 2001) and proline but definitive evidence on their role and importance has not yet been produced. Survivor genotypes from populations of white clover grown in cold sites in Europe were found to have higher levels of unsaturated fatty acids in their stolon tissue than the original populations, suggesting that this trait is of adaptive significance in cold climates (Collins et al. 2002). Low-temperature interacts with other stresses that affect plant survival such as grazing pressure and successful efforts to produce material with enhanced winter hardiness under farm conditions have incorporated this complexity into their selection regimes (Rhodes et al. 1994). Wachendorf et al. (2001) showed that the behaviour of white clover including over-wintering and growth in spring can be successfully modelled. On acid soils, forage legumes may have their productivity limited by a lack of aluminium tolerance. Selections have been made in the field (Caradus et al. 2001) and using artificial tests (Voigt and Staley 2004) with limited success. Although white clover is a highly heterozygous outbreeder with considerable variation available for the improvement of many traits, there are some desirable attributes where this is not the case. Thus, only limited variability is present for drought tolerance, a characteristic which has long been a major objective in more arid areas (e.g. parts of Australia and New Zealand) and is becoming of increasing importance in other parts due to the likely effects of climate change. Barbour et al. (1996) showed differences between ten white clover cultivars with respect to their response to water stress but Brink and Pederson (1998) found little variation in response to a water gradient between six lines. Field studies have also shown that drought, in combination with other stresses and influenced by management, can have marked effects on plant survival and these effects differentiate between plant populations (Jahufer et al. 1995).

5.3 Pest and Disease Resistance

The major pests of white clover in UK pastures are slugs, Sitona weevil and stem nematode (*Ditylenchus dipsaci*). Slugs can cause considerable damage particularly in re-seeds or over-sowing. In these circumstances they are often controlled by pellets. Slugs are repelled by cyanogenic glycosides and the level of cyanogenesis in a variety can be controlled by the incorporation of a number of acyanogenic genotypes in synthetic development. Considerable efforts have been made with respect to improved resistance to stem nematode, *D. dipsaci*. Although it is difficult to obtain definitive evidence for its importance as a factor leading to reduced yield or persistence there are a number of observations both field based and experimental that suggest nematode infestation is significant in reduced performance and can occasionally cause more pronounced declines in stand density. In New Zealand and Australia attention has focused on clover cyst nematode and resistant varieties have been produced (Mercer et al. 2008). However, Sitona weevil has emerged in recent years as the major threat to white clover in NZ pastures. Viruses, in particular white clover mosaic virus (WCMV), have been shown to be major causes of yield reduction in NZ pastures (Dudas et al. 1998) but their importance elsewhere is not well defined. With respect to fungal pathogens, root rot has been the focus of considerable efforts involving greenhouse and field evaluation, particularly in Canada. In Europe, *Sclerotinia trifoliorum* is considered to be the main fungal threat and resistance screens have been developed.

5.4 Symbiotic Interactions

Clearly the most notable feature of forage legumes is their ability to fix atmospheric nitrogen through the root nodules they form in symbiotic association with Rhizobia bacteria. In white clover, a number of estimates of the amount of nitrogen fixed have been carried out. Thus, for instance, Ledgard et al. (1999) reported values of 99–231 kg N/ha/yr from dairy farmlets in New Zealand. Figures reported for white clover are in general greater than those for red clover at similar biomass levels, mainly because of the higher N content of white clover. However, symbiotic dependence measured by isotope dilution techniques tends to be lower for white than for red clover. Considerable dependence on fixed N can be observed for white clover in mixed swards even with high levels of mineral N although the relative contribution of fixed N may be reduced under grazing (Eriksen and Hogh Jensen 1998), possibly due to the contribution of mineralised N derived from animal returns. The major variable seems to be the clover content of the sward rather than fixation-related processes per se. This may partly explain why relatively little effort has been put into breeding for improved fixation (Crush and Caradus 1996). A contributing factor is the difficulty of implementing an efficient screening system for use on large numbers of plants. Similarly, the transfer of fixed nitrogen from clover to the companion grass

has been shown to be an important factor in maintaining total sward performance. McNeill and Wood (1996) estimated the annual nitrogen fixation by white clover in the UK at 155 kg N/ha. Of this they calculated that approximately 28% was transferred to the ryegrass companion in mixed swards and that this in turn represented about 29% of the total nitrogen content of the ryegrass. Transfer of fixed N can occur through decomposition of below ground parts of the legume, through nutrient cycling mediated by root herbivory (Murray and Clements 1998) and through animal excreta. Little is known about the details of the former process and it is difficult to quantify. Thus, although differences between varieties have been noted (Laidlaw et al. 1996) this trait has proven difficult to reduce to clear selection criteria and has not been incorporated in plant breeding programmes to any great extent. There is a need for a greater mechanistic understanding of the transfer process and the important plant traits contributing to them. Parsons et al. (1991) studied the cycling of N in grass/white clover swards from the standpoint of its effects on species composition and this has subsequently been developed into models with N cycling as a driver of changes in grass/clover balance (e.g. Wu and McGechan (1999). The impact of clovers on the nitrogen cycle is not restricted to fixation and transfer to the grass companion. Apart from nitrogen cycling on the farm scale through the use of manure, N becomes available for the crop succeeding a forage legume in a rotation. This is strongly influenced by the extent of rhizodeposition (Hogh-Jensen and Schjoerring 2001. The use of flowing solution culture (FSC) or hydroponics has proved valuable in dissecting the physiological interactions between N fixation and availability of other N sources.

5.5 Compatibility with Companion Grasses

Compatibility with companion grasses, particularly perennial ryegrass, *L. perenne* L., allowing high total swards yields, has long been a major breeding objective (e.g. Annichiarico 2003) in white clover, and a number of studies have been carried out to disentangle some of the main traits involved in both species (e.g. Caradus and Mackay 1991). A general review of white clover management in mixed swards is given by Frame and Laidlaw (1998). Clearly, differences in response to temperature and competition for light play major roles (Robin et al. 1994) but below ground interactions are also likely to be important (Collins and Rhodes 1994, Caradus and Woodfield 1998). Some modelling-based approaches to grass/clover interactions have emphasised the importance of nitrogen build-up and its feedback inhibition on fixation in affecting the 'cycling' of relative grass and clover yields over time. However, in practice a largely empirical approach has been taken and selection based on performance over a number of years, sometimes with a range of different companion varieties, in mixed plots under cutting or a management regime more closely simulating likely use of white clover on the farm (Evans and Williams 1987, Gilliland 1996). Mixtures or blends of white clover varieties with different characteristics have been employed to increase yield stability (Williams et al. 2003).

5.6 Animal Nutrition

In recent years increasing emphasis has been given to the animal production consequences of grazing grass/clover swards (e.g. Frankow-Lindberg and Danielsson 1997). The factors important for the utilisation of forage legumes by the ruminant animal were discussed by Beever and Thorp (1996) and the breeding of forages for increased nutritional value was reviewed by Casler and Vogel (1999) and Casler (2001). Quesenberry and Casler (2001) note that in general forage quality traits are less sensitive to G × E interactions than agronomic traits such as yield. Evidence is accumulating that incorporation of a substantial forage legume component in the diet, both grazed and ensiled, can enhance meat and milk quality. The difference between a forage-rich and particularly clover-rich diet and concentrates in terms of enhanced levels of beneficial PUFAS in meat and milk is now well established. Furthermore, Al-Mabruk et al. (2000) showed that the levels of alpha tocopherol (vitamin E) found in milk from Holstein dairy cows were higher following a diet with a high forage component than one based on concentrates.

An approach to reduction in bloating propensity is that of reducing the rate of cell wall degradation. The degradability of protein in the rumen is important not only in terms of bloat but also as a factor influencing the efficiency of N use. Proteolysis in the rumen is a key process in the utilisation of forage legumes and recent evidence suggests that this may be mediated by plant processes as well as rumen microbes (Zhu et al. 1999, Kingston-Smith et al. 2003). Cyanogenesis potential in white clover is a concern in some countries with respect to the effects on large herbivores and this is given consideration in a number of breeding programmes (e.g. Crush and Caradus 1995). Increasingly sophisticated approaches to analysing grazing behaviour have contributed greatly to our understanding of the differences in nutrition and performance in comparison with silage and/or concentrate feeding or cut and carry. For instance, Orr et al. (1997) showed that sheep show a marked diurnal shift in their preference for clover (mornings) and grass (later in the day).

5.7 Environmental Impacts

Clovers are widely regarded as ‘environmentally friendly’ particularly as their nitrogen fixation reduces the need for nitrogenous fertiliser and their high protein content, digestibility and palatability means that lower amounts of concentrates have to be imported on to the farm. These features are at the heart of the beneficial economic impacts of clover use. Jarvis et al. (1996) in a systems synthesis study of dairy farms found that use of white clover, especially at relatively low clover contents, was an effective approach to reducing nitrogenous losses. However, there was a cost to production and losses per livestock unit did not differ markedly from those under some alternative management systems. Parsons et al. (1991) showed that 80% of the sheep carrying capacity of a grass sward receiving 420 kg N/ha/yr could be maintained with a white clover content of 5% or less fixing only 24 kg N/ha/yr,

leading to a marked reduction in nitrogenous losses. Jarvis et al. (1996) showed that 66% of the support energy for grassland management on a dairy farm came from fertiliser production and that this could be more than halved by the use of white clover. However, a concern with the use of any legume is the fate of fixed N. Davies et al. (2001) considered this in a comparison of ploughed grass and grass/clover swards and Ledgard et al. (1999) studied losses under grazing by dairy cows. N leaching can occur under grass/clover swards and in extreme circumstances this is comparable to swards fertilised at rates commonly used in agricultural practice. However, as long as N fertilisation does not exceed 300 kg/ha/ yr, N leaching from grazed grassland does not lead to a violation of the EU Nitrate Directive (Benoit and Simon 2004), and grass-clover swards are not more prone to nitrate leaching than pure grass swards (Loiseau et al. 2001). At low to moderate N fertilisation (up to 150 kg/ha/yr^{-1}), nitrate concentration in the soil solution can become a problem only if the clover content of the sward reaches 80% or more (Nyfeler, 2009), which is very rarely the case in agricultural practice. Therefore, a reasonable management of grass-clover swards is sufficient to limit N leaching, and there is no immediate need to consider the role of germplasm improvement in reducing such losses.

Phosphorus (P) requirements for good clover performance are relatively high and work has been carried out, particularly in New Zealand, to select lines which can yield well at lower levels of P fertilisation (Caradus 1994). P pollution from farmland is widely regarded as a growing environmental problem and one which is likely to be addressed by regulation in many parts of the world (e.g. EU Water Framework Directive). It seems likely that selection for growth at low P will increase in importance and several approaches may prove viable in addition to direct selection (Figure 1). The extent of inorganic P incorporation into organic compounds shows genetic variation (Caradus et al. 1998) and may be an important selection

Fig. 1 Hydroponics system to enable analysis of P uptake and loss from white clover (Photo Athole Marshall)

criterion for future studies. The symbiosis between white clover and arbuscular mycorrhizal fungi (AMF) or vesicular arbuscular mycorrhizae (VAM) has been explored with a view to increasing the efficiency of P uptake (e.g. Crush 1995), although the role and importance of this association on fertilised agricultural soils is unclear. There is evidence that this symbiosis plays an important role in P uptake, in protection against pathogens and in improving the drought tolerance of the host plant. Inbred lines of white clover have been used to unravel aspects of the plant genotype–AMF interaction (Eason et al. 2001). Secretion of phytase under P deficiency is seen in many plant species and may be a future target of forage improvement (Li et al. 1997).

Over the past decade the effects of elevated ozone levels on crop plants have been the subject of increasing research. White clover has been developed as an 'indicator' species for ozone bio-monitoring (e.g. Fumagalli et al. 2003). Clearly, white clover genotypes differ in ozone sensitivity; however, no targeted breeding efforts to improve tolerance have been undertaken so far. A topic of growing concern is methane emissions from livestock, particularly cattle, and this is a likely target for efforts with respect to changes in plant composition that may reduce emissions (Ulyatt et al. 1997). There is evidence that white clover has a beneficial effect on soil quality (Mytton et al. 1993). It has been reported that the changes in soil structuring brought about by white clover resulted in improvements in water percolation rate (i.e. the soil became more freely drained), and in the extraction by plants of nutrients from the soil. Holtham et al. (2007) also reported evidence of local structuring of soil around white clover roots and greater drainage of water through soil cores under white clover than under perennial ryegrass monocultures.

6 Breeding Methods

6.1 Classical Approaches

Approaches based on mass phenotypic or recurrent selection, typical for outbreeding species, have been employed in forage legume breeding programmes. These have utilised field, glasshouse and controlled environment-based schemes of assessment for important traits. In general assessment of single (spaced) plants in rows is useful for a general characterisation of new germplasm resources and particularly for leaf size (at an early stage in the breeding cycle) and evaluation of DUS characters (towards the end of the cycle). However, for agronomic characters and performance effective evaluation is carried out in swards with the companion grasses over a period of at least 3 years and with an appropriate management involving either sheep or cattle grazing depending on leaf size. Variety development in general follows the route of developing synthetics based on a small number of mother plants.

An important approach to a greater insight into the genetic control of key traits in white clover has been the development of self-fertile inbred lines. Attwood (1942)

described the SI system in this species and Yamada et al. (1989) developed inbred lines through recurrent selection. Michaelson-Yeates et al. (1997) carried out a number of generations of inbreeding with several different lines and analysed the degree of heterosis in crosses between them. The genetic distance between lines was subsequently estimated (Joyce et al. 1999) and this information was used in the choice of parents for the first molecular genetic mapping family in white clover (see later). The highly inbred lines have also proved a very useful tool in other work and have facilitated studies, for example characterising variation in N fixation and N uptake in flowing solution culture (Michaelson-Yeates et al. 1998). Similar techniques have been applied to population variability in managed populations (e.g. Gustine and Huff 1999) and breeding populations. Application of these approaches has perhaps not fully capitalised on the potential to bring together heterotic groups of different origins as described by Brummer (1999).

In some cases, morphological markers for traits of agronomic importance have been utilised and leaf marking has been used as a morphological marker and DUS character (Bortnem and Boe 2002) and to monitor changes in white clover morphology in mixed swards (Fothergill et al. 2001). Breeding programmes have been aided by the development of improved analytical techniques. The use of near-infrared reflectance spectroscopy (NIRS) for measurement of chemical composition (Berardo 1997) and clover content in mixed swards (Wachendorf et al. 1999) has been an important development of the last decade. For many important traits there remains, however, the problem of developing effective screens. Voigt and Staley (2004), for instance, demonstrated that a soil on agar method was not effective for selecting genotypes with superior acid soil resistance. Where artificial screens do give enhancement for traits related to edaphic stresses these may not be maintained in the long term in the field (Caradus et al. 2001). Where variation for a specific trait is not present within existing germplasm then traits have been introduced by interspecific hybridisation. Hybrids between white clover and the annual, profuse flowering ball clover (*Trifolium nigrescens* Viv.) have been developed, using conventional crossing techniques, with the objective of introgressing reproductive traits into white clover (Marshall et al. 2002). Transfer of clover cyst nematode resistance from *T. nigrescens* into white clover has also been a target (Hussain et al. 1997).

Phenotypic selection for improved drought tolerance or for yield under drought stress conditions is widely accepted as difficult. This is because occurrences of drought stress in natural environments are highly variable in their timing, duration and severity, making it difficult to identify traits that confer a predictable advantage across stress environments. Direct selection for drought tolerance has been carried out in the field and indirect methods have also been used, but success has been limited. Where insufficient genetic variation is available to achieve any significant improvements in drought resistance from within a species, increasingly new allelic variants are being sought from wild relatives adapted to drier environments. Backcross hybrids have been produced between white clover and the more drought-tolerant Kura or Caucasian clover (*T. ambiguum* M. Bieb.) with white clover as the recurrent parent and show considerably enhanced drought resistance compared to the white clover parent (Marshall et al. 2001). The basis of this enhanced drought

resistance is not clear; however, differences in stomatal density and in root density throughout the soil profile have been identified between parental species and hybrids. An alternative approach has been the development of a 'fertile bridge' between *T. ambiguum* and white clover, enabling the transfer of traits between the two species without the use of embryo rescue (Hussain and Williams 1997).

6.2 Molecular Markers

The use of molecular markers offers considerable potential advantages for the breeding of perennial species where evaluation of advanced lines in plots takes at least 3–4 years and where studies involving animals or impacts on the environment are time consuming and expensive. Significant progress has been made since the development of the first white clover genome map (Jones et al. 2003). In particular, QTL for important traits have been identified (Cogan et al. 2006, Abberton et al. 2009) and marker-assisted selection utilised for improvement in seed yield (Barrett et al. 2009). Further progress will be facilitated by resource development including a bacterial artificial chromosome (BAC) library (Febrer et al. 2007), the use of *T. occidentale* as a stoloniferous 'model' species (Williams et al. 2009) and the dissection of subgenome-specific markers (Lawless et al. 2009). Markers for the introgressed traits in the *T. repens* × *T. ambiguum* and *T. repens* × *T. nigrescens* hybrids have been developed using a bulked segregant approach with amplified fragment length polymorphism (AFLP) markers (Abberton et al. 2003). Molecular markers have also been used to identify genetic relationships in parental material and for germplasm characterisation more widely in breeding and natural populations (e.g. Gustine and Huff 1999, Kölliker et al. 2001) and between cultivars. Translation of the information, tools and resources arising from studies of the model legumes, *Medicago truncatula* and *Lotus japonicus*, will be an important avenue feeding into clover germplasm improvement in the future. Such developments will also be aided by genomic studies currently been carried out on red clover (*Trifolium pratense* L.) which is more closely related to white clover and, since it is diploid and has a much smaller genome size, is more amenable to genomic analysis.

6.3 Genetic Modification

White clover transformation is well established although at relatively low efficiency (Webb 1996, Voisey et al. 1994). Ding et al. (2003) reported on methods devised to increase transformation efficiency in both *Trifolium* and *Medicago* species and Lin et al. (2003) showed that promoters from *Arabidopsis* could function in an organ-specific and inducible way in white clover. Considerable emphasis has been given to transgenic approaches to pest and disease resistance. The first transgenic white clover to progress to field trials (in New Zealand) contained viral coat protein resistance to white clover mosaic virus (WCMV) (Dudas et al. 1998). Progress in the

production of transgenic white clover with enhanced pest and disease resistance was reviewed by Voisey et al. (2001). A key objective of research in transgenics on white clover has been the production of condensed tannins in the leaves. Condensed tannins have been implicated not only in bloat prevention but also as contributing to the anthelmintic effects of forages such as *Lotus corniculatus* or sainfoin. Marley et al. (2003) demonstrated this in sheep although the mechanism is unclear and may involve stimulation of the immune response rather than a direct effect of tannins per se. The ability to manipulate that part of the pathway leading to the production of tannins, which is in common with anthocyanin production, was shown by Mouradov et al. (2009). Attempts to improve protein quality have included the expression of the pea albumin 1 gene in white clover (Ealing et al. 1994) and delta zein, a sulphur-rich maize storage protein (Sharma et al. 1998). Jenkins et al. (2002) reported fructan formation in white clover expressing a fructosyl transferase from *Streptococcus*. Other applications of transgenic approaches in white clover include delayed leaf senescence following the introduction of the isopentyl transferase gene involved in cytokinin biosynthesis (Spangenberg et al. 2001). The key question of the stability and background dependence of transgene expression was addressed by Scott et al. (1998). The potential for transgenic approaches in future germplasm improvement was considered by Spangenberg (2005).

7 Seed Production

7.1 Breeding for Seed Production

Although bred predominantly for forage production and forage quality, the ability to produce reasonable amounts of seed is important for the commercial success of a variety. Relatively few studies have been carried out in recent years to improve the seed yield potential of clovers. In white clover, the number of ripe inflorescences is the main seed yield component and closely correlated with seed yield (Jahufer and Gawler 2000) and significant genotypic variation for reproductive traits exists (Cain et al. 1995, Jahufer and Gawler 2000). In breeding programmes seed production potential of white clover varieties is measured in field plots (Figure 2).

Inflorescences are produced at nodes on the developing stolon, but not all stolon nodes are reproductive and a balance between reproductive nodes and nodes that develop secondary stolons is important to maintain the persistency of a variety. Annicchiarico et al. (1999) reported that persistence as predicted by stolon density was negatively correlated with seed yield and DM yield. Selection of traits which improve seed yield without impairing agronomic performance has been explored. Marshall (1995) reported that selection for peduncle (flower stalk) strength improved inflorescence survival and increased seed yield significantly. An appropriate response to changes in day length, particularly in terms of the balance of vegetative and reproductive growth, is an important consideration in the use of germplasm accessions (Quesenberry and Casler 2001).

Fig. 2 Comparison of seed yield potential of white clover varieties in field plots (Photo Athole Marshall)

7.2 Seed Production

White clover is a bee-pollinated species. Pollination in the early stages of variety development is often carried out in bee-proof isolation chambers using alfalfa leaf cutter bees (*Megachile rotundata*) or in polythene tunnels (Figure 3).

In field crops white clover is pollinated by honey bees (*Apis mellifera* L.) or bumble bees (*Bombus* spp.); therefore, seed production needs to be located where there is a plentiful supply of indigenous pollinators or where there are sufficient

Fig. 3 Seed production of white clover in a polythene tunnel (Photo Athole Marshall)

hives available. Certified seed production requires adequate isolation from potential sources of cross-pollination and minimum isolation distances are prescribed in seed regulations. This distance can vary from 50 to 200 m depending on several factors (size of crop, generation of seed production, type of crop to be isolated), including country of production. The largest producer of certified white clover seed is New Zealand with Denmark also producing significant quantities. In 2006, the most recent year for which full statistics are available, 5287 t were produced in New Zealand and 2806 t in Denmark accounting for more than 80% of the world-certified seed production.

In common with many other forage species white clover seed production is influenced by weather conditions. Climatic conditions at pollination and harvest have a significant effect on white clover seed production. Excessive rainfall can have a detrimental effect on pollinator activity and lead to excessive vegetative growth which can reduce flowering and the effectiveness of harvesting. Seed yields vary considerably from year to year and consequently seed production is predominant in countries where climatic conditions are more suitable for production. Seed crop management systems have been extensively researched and reviewed (Marshall et al. 1997). Research has been carried out to identify methods of controlling leaf production and promoting reproductive growth. In climates where rainfall is limited controlled moisture stress can promote inflorescence production.

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Minor Legume Species

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1 Introduction

The choice of what is a 'minor' legume species to be treated in this chapter was certainly arbitrary and questionable, especially when considering the array of legume species used worldwide as forage crops. A forcedly limited number of species are described here which were considered important among the forage legumes once lucerne (*Medicago sativa* L.), white clover (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.) were excluded. The species chosen are either outstandingly important in a localised geographic area while being successfully introduced elsewhere, such as the annual self-reseeding species, or they have a generalised moderate importance across a wide range of areas, such as berseem clover (*T. alexandrinum* L.). Other species were included which have really become 'minor' in their once important areas of cultivation – such as sulla (*H. coronarium* L.), birdsfoot trefoil (*L. corniculatus* L.) and sainfoin (*O. viciifolia* Scop.) in central and southern Europe – but their introduction in other areas is maintaining their relevance worldwide. Furthermore, there is a renewed interest on them, given their recognised role in enhancing the sustainability of extensive, low-input livestock systems in pedo-climatically unfavourable environments.

2 Annual Self-Reseeding Species

2.1 Biological and Agronomic Features

In an agronomic sense, the term self-reseeding applies to pasture legume species in which the produced seeds determine the ability to self-regenerate dense stands one life cycle after the other. Although primarily depending on seed yield, their self-regeneration is also driven by the presence of physiological mechanisms preventing a prompt germination of all mature seeds, enabling, therefore, the formation

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of a seed bank in the soil. The most important of these mechanisms is seed coat impermeability, termed as hardseededness (Quinlivan 1971).

The agronomically relevant annual self-reseeding legumes include subterranean clover (*T. subterraneum* L. *sensu lato*) and annual medics (*Medicago* sp.). Both originated from the Mediterranean Basin (Katznelson 1974; Lesins and Lesins 1979), but got an important role as cultivated species in Australia, where they were fortuitously introduced during the 19th century and became a common component of a ley-farming system specifically adapted to annual legumes. This is an integrated cereal–livestock production system, where the annual legume is alternated with the cereal crop, thus self-regenerating the pasture from the seed bank after each cereal 'phase' and providing a good part of the nitrogen needs to the cereal.

More recently, the annual self-reseeding legumes have also raised an agronomic interest in the Mediterranean Basin. In North Africa and West Asia, attention has been paid to the possible introduction of ley-farming, with the annual medics replacing fallow in rotation with a cereal crop. The attempts to introduce ley-farming, however, were often unsuccessful as a possible result of lack of adaptation of Australian selections to the prevailing conditions in the region (Cocks 1992a). Extensive livestock systems in southern Europe rely widely on permanent pastures and rangelands, which could be much improved by the exploitation of annual self-reseeding legumes such as subterranean clover (Piano 1989).

A more recent and alternative use of the annual self-reseeding legumes in southern Europe and the USA is as cover crops in vineyards and orchards (Pecetti et al. 2007). Their cycle does not overlap substantially with that of the woody species (particularly of grape vine) and they do not compete for water resources with the main crop.

All annual medics and subterranean clover are autogamous, with cleistogamous flowers and self-tripping mechanism (Katznelson and Morley 1965; Lesins and Lesins 1979). The possibility exists, nonetheless, that occasional outcrossing also occurs in these species. Marshall and Broué (1973) estimated the outcrossing rate of Australian populations in subterranean clover as low as 0.15%. Even a very limited amount of outcrossing, however, has probably been of great relevance to the evolution of autogamous self-reseeding legumes (Katznelson and Morley 1965). The variation released by occasional hybridisation can be subsequently fixed by selfing, and made available to the natural selective pressures (Cocks 1992b). Natural populations of subterranean clover are found to be formed by clusters of several genetically distinct strains (Piano 1984).

2.2 Breeding Efforts

Breeding of both annual medics and subterranean clover began in Australia around the mid-20th century, when their great potential as pasture species was realised. In recent decades, dedicated breeding programmes were also started elsewhere, particularly in southern Europe. Because of their mating system and huge germplasm variation already existing in nature, the breeding of these species has mostly been based on a pure-line selection from natural populations. In Australia,

the earlier selection largely relied on strains which had become naturalised to that country (either fortuitously introduced there as such, or originated from the above-mentioned occasional crossbreeding events), whereas more recently the selection also relied on germplasm purposely collected in centres of origin/diversification of those species, particularly in the Mediterranean Basin (Nichols et al. 1996). By the pedigree selection method, crossbred cultivars were released from the 1970s onwards, often obtained for specific targets such as tolerance to diseases and pests. Selection methods which combine the advantages of the pedigree and those of the bulk method were subsequently set up to enhance the effectiveness of selection for adaptation (Nichols 1993). In recent years, the breeding of annual self-reseeding legumes, as that of many other species, is being concerned with innovative approaches and methods, such as molecular marker applications and genetic transformation.

2.3 Annual Medics (*Medicago* sp.)

2.3.1 Origin and Systematics

Lesins and Lesins (1979) classified 33 annual species within the genus *Medicago*. They are naturally distributed over a wide range of environmental conditions throughout the Mediterranean region. Piano and Francis (1992) reported a thorough description of the eco-geography of annual medics and discussed related aspects of plant introduction and breeding. In addition to differences among species, intra-specific variation also occurs in response to soil and climate factors.

The genetic diversity existing among and within annual medics has certainly not been adequately exploited yet. The 31 cultivars listed in the Register of Australian Herbage Plant Cultivars, 2007 edition (www.pi.csiro.au/ahpc/legumes/legumes.htm), encompass only 7 species, namely strand medic (*M. littoralis* Rohde), murex medic (*M. murex* Willd.), gama medic (*M. rugosa* Desr.), snail medic [*M. scutellata* (L.) Miller], disc medic [*M. tornata* (L.) Miller], barrel medic (*M. truncatula* Gaertn.) and burr medic (*M. polymorpha* L.). Apart from these seven species, only one cultivar each of wheel medic (*M. rotata* Boiss.) and sphere medic (*M. sphaerocarpors* Bertol.) and one cultivar of hybrid origin *M. littoralis* × *M. tornata* have been selected. Outside Australia, cultivars were selected in *M. polymorpha* in Italy and France, and in *M. rigidula* (L.) All. and *M. truncatula* in France. These three species are among the few 'key' species identified by Piano and Francis (1992) for use in the Mediterranean Basin because of their widespread distribution as regards soil and climate and their recognised grazing tolerance. *M. polymorpha* appeared as the species with the largest environmental range of adaptation.

2.3.2 Genetic Resources

A large number of regional germplasm collections have been established, including eco-geographic information. The Australian *Medicago* Genetic Resource Centre in

Adelaide, Australia, maintains the world's largest collection of annual and perennial *Medicago* species, with over 25,000 accessions. In Europe, large collections of annual medics have been established in Le Magneraud, France, and Badajoz, Spain. The ECPGR annual *Medicago* Database is maintained by Servicio de Investigación y Desarrollo Tecnológico in Spain and included 3043 accessions from eight holding institutes in 2007.

Populations of naturalised strains may be found in situ in Australia but colonisation was restricted to neutral-alkaline soils due to misadaptation of the pioneer species to acidic soils. In the Mediterranean Basin, medics are more widely distributed in terms of soil pH, and species such as *M. murex*, *M. polymorpha*, *M. arabica* (L.) Huds. and *M. soleirolii* also extend onto acidic soils (Piano et al. 1982). *M. murex*, in particular, is interesting for its ability to colonise and establish good nodulation in those soils. Acid-tolerant rhizobial strains were isolated from adapted medic species in acidic soils of the Mediterranean region and supported the introduction of varieties of these species into acidic soils of Australia (Howieson and Loi 1994; Gillespie 2006).

2.3.3 Major Breeding Goals and Achievements

Selection criteria of medics may largely differ between the use for ley-farming systems in Australia and pasture improvement in the Mediterranean Basin. In the latter exploitation system, longer growing season, greater frost tolerance, higher seedling vigour and better competition with weeds are required, while very high hardseededness, which is a major requirement for ley-farming, should no longer be a must (Prosperi et al. 1996).

Frost susceptibility has often been the main cause of failure of Australian commercial species and cultivars in the Mediterranean Basin, as was shown for 19 Australian cultivars grown in northern Italy (Pecetti et al. 2007). Conversely, indigenous medic species such as *M. rigidula* and *M. aculeata* Gaertn. (syn. *M. doliata* Carmign.) were found to be tolerant to low temperatures.

The generally short life cycle of medics enables them to escape severe spring–summer drought. However, variability exists in their natural habitats, which extend from sub-desertic to high-rainfall areas (Piano and Francis 1992). Among the agronomically relevant species, *M. littoralis*, *M. truncatula* and *M. tornata* are very frequent in dry environments. The species reportedly enduring the lowest rainfall are *M. laciniata* (L.) Mill. and *M. radiata* L. Adaptive strategies of persistence may be derived from the distribution of such species. High frequency of species with many, small seeds per pod (their advantage lying in the lower need of water for germination and faster maturity rate than larger seeds) and high hardseededness have been reported in dry areas (Ehrman and Cocks 1990). Breeding of Australian commercial cultivars has generally been carried out under semi-arid conditions (Crawford et al. 1989).

Species common on clay to clay–loam soils [e.g. *M. rugosa*, *M. aculeata*, *M. scutellata*, *M. intertexta* (L.) Mill. or *M. ciliaris* (L.) Krock.] are generally

large-seeded (Piano and Francis 1992), a feature possibly reflecting some advantage for seedling emergence and root penetration in soils subject to compaction.

Lines resistant to the foliar diseases *Phoma* black stem (*Phoma medicaginis* Malbr. et Roum.) and pepper spot [*Leptosphaerulina trifolii* (Rostovzev) Petr.] were identified in *M. sphaerocarpos*, *M. murex*, *M. truncatula* and *M. soleirolii* (Barbetti 2007). *M. ciliaris* was reported to be symptomless and tolerant to the alfalfa mosaic virus.

Several medic cultivars resistant to different species of aphids have been selected in the last decades through ad hoc crossing programmes. Some sources of resistance to other foliage insects (alfalfa weevil, *Hypera postica* Gyllenhal; potato leafhopper, *Empoasca fabae* Harris) or seed chalcids (*Bruchophagus roddi* Gussak) were reported in glandular-haired species (e.g. *M. rugosa*, *M. scutellata*, *M. minima* Bart., *M. blancheana* Boiss., *M. disciformis* DC, *M. rigidula*, *M. ciliaris*, *M. rotata*) (Sorensen et al. 1988).

Although forage quality in the sense of nutritive value did not represent a major target in Australian breeding programmes, an emphasis was placed in selecting cultivars with low levels of anti-nutritional factors, such as phytoestrogens. Coumestrol and other methylated coumestans were isolated from medics, which may cause reproductive disorders in grazing ewes due to their estrogenic activity (Kelly et al. 1976). The commonly recorded levels of coumestans are considered to be of little biological significance, but fungal diseases and viruses may cause a considerable increase of coumestan concentrations in dry stems and pods (Barbetti 2007).

Saponins raised growing interest because of their possible negative (e.g. anti-nutritional, hemolytic) or positive (antifungal, insecticidal, nematicidal, phytotoxic) implications (Hostettmann and Marston 1995). Tava and Avato (2006) extensively reviewed the chemical structure and biological activity of saponins from *Medicago* species, including several annual medics.

Besides being one of the most widely grown medic species worldwide, *M. truncatula* has been established as the 'model plant' of the Trifolieae tribe, and of the genus *Medicago* in particular, for studying legume biology, genetics and genomics (Cook 1999), becoming a case in the last years with little equal for such a successful plant species in research applications (see, for instance, the handbook available online at www.noble.org/MedicagoHandbook).

2.4 Subterranean Clover (*Trifolium subterraneum* L. *sensu lato*)

2.4.1 Origin and Systematics

The subterranean clover complex includes three subspecies, namely *T. subterraneum* subsp. *subterraneum* L., *T. subterraneum* subsp. *brachycalycinum* Katzn. et Morley and *T. subterraneum* subsp. *yanninicum* Katzn. et Morley. The species is thought to have originated from Turkey, and it is widespread in the Mediterranean Basin and western Europe (Katznelson and Morley 1965). The three subspecies are rather easily recognisable by their morphophysiology, karyotype, isozyme

patterns and polymorphism for molecular markers, and although often growing sympatrically (Piano et al. 1982) they are almost completely intersterile, suggesting that a speciation process is taking place in the complex (Katznelson and Morley 1965).

The Australian naturalised strains, from which new cultivars were selected for decades, belong almost exclusively to subsp. *subterraneum*. In the Mediterranean region, the subsp. *brachycalycinum* occurs as frequently as the subsp. *subterraneum* (Piano et al. 1982). The subsp. *yanninicum* is the least common one and much more restricted geographically. For a long time it was believed to occur only in Greece and in some areas of the Balkans (Morley and Katznelson 1965), but sporadic findings were reported from Spain (Katznelson 1974) and several populations of this subspecies were collected in Sardinia (Piano et al. 1982).

Each subspecies has a characteristic suitability to specific soil and environmental conditions (Piano et al. 1982; Nichols et al. 1996). The subsp. *subterraneum* is best adapted to light, slightly acidic to neutral soils. The subsp. *brachycalycinum* is often found in heavy, cracking or self-mulching soils, with neutral to alkaline pH. The subsp. *yanninicum* prefers neutral to slight acidic soils; it is particularly adapted to waterlogged conditions, but it also grows well in soils with good drainage.

2.4.2 Genetic Resources

Extensive collection of genetic resources from the Mediterranean centre of diversity of the species has increasingly been seen as a means of providing valuable materials to breeding programmes, both for Southern European countries and Australia (Piano 1984; Nichols et al. 1996). Being an autogamous species, the collection of native populations and the singling out of the pure lines forming each population result in the fast availability of homogeneous genetic material for the breeding. Identification of individual pure lines within a population sample has been made possible by the use of combinations of peculiar morphological descriptors (e.g. leaflet markings, anthocyanin pigmentations, pubescences), which often characterise unequivocally each genotype (Piano 1984).

A world collection of subterranean clover germplasm with several thousand accessions has been assembled at the Australian *Trifolium* Genetic Resource Centre in Perth, while large regional collections have been established in Badajoz, Spain, and Lodi, Italy.

2.4.3 Major Breeding Goals and Achievements

A milestone in the breeding of subterranean clover in Australia was the establishment of the National Subterranean Clover Improvement Programme, which defined selection objectives in relation to novel and diversified requirements across the country and released a range of new varieties. The Register of Australian Herbage Plant Cultivars, 2007 edition lists 37 cultivars selected in that country. In recent

years, locally bred cultivars were also developed in Southern European countries such as Spain and Italy (González López 1994; Piano et al. 1997).

The introduction of Australian cultivars into Mediterranean pastures was often prone to failure. These failures could be mostly ascribed to an incorrect use of different subspecies in relation to their edaphic specialisation or to the choice of cultivars of inappropriate maturity class. The success of a cultivar, which is mostly expressed in terms of persistence, i.e. long-term self-regeneration ability rather than forage yield per se, derives from a set of characters, the particular combination of which must be specific to the environment of utilisation. The assessment of the relationships between the variation in key adaptive traits of natural populations and the ecological characteristics of their sites of origin has provided selection models for developing new cultivars in given regions (Piano et al. 1996). The main selection criteria pursued in major breeding programmes (Nichols et al. 1996; Piano and Pecetti 1996) can be summarised as follows.

Maturity grading. In a Mediterranean climate, the onset of spring–summer drought terminates the growing cycle of annual self-reseeding legumes. Maturity grading is a complex trait including time of commencement of flowering, duration of flowering and rate of seed development. The primary requirement for adaptation is that maturity in a given environment is early enough to allow adequate seed setting before the beginning of the adverse season. Early flowering and rapid seed formation confer an advantage for seed production in environments with short growing season (Piano et al. 1996) while the role of flowering duration under stress is controversial.

Selection of cultivars with very diversified maturity grading in function of the target environment has not been a major problem (Nichols et al. 1996), with the possible exception of subsp. *brachycalycinum* where lower variation is available, especially towards early variants. Development of adapted early to mid-season cultivars in this subspecies is being pursued through ad hoc selection programmes (de Koning et al. 1996). In the subsp. *subterraneum*, the genotypic range of variation for maturity can extend for up to 70 days in flowering time even within the same population (Piano 1984). Apparently, a mechanism of 'disruptive selection' (Hayward et al. 1993) helped these populations to cope with the unpredictable seasonal climatic fluctuations and assure continued persistence by maintaining high intra-population variation.

Burial ability. The peculiar geotropy of the apex of the floral peduncle determines the active burial of the reproductive structures and the underground maturation of the seeds in a false fruit called 'burr', formed by a proliferation of sterile calyces wrapping usually three to four uni-seeded pods (Figure 1).

Lack of burial has a detrimental effect on seed yield, seed viability, hardseededness and seedling re-establishment (Quinlivan and Francis 1971). Great variation is found among genotypes in strength of burr burial, particularly in subsp. *subterraneum* (Piano and Pecetti 1995; Nichols et al. 1996). Differences in burr burial ability are evident between the subspp. *subterraneum* and *brachycalycinum* (Francis et al. 1971; Piano and Pecetti 1995), which reflect their edaphic adaptation and the partial modification of the typical reproductive mechanism developed by the latter. The

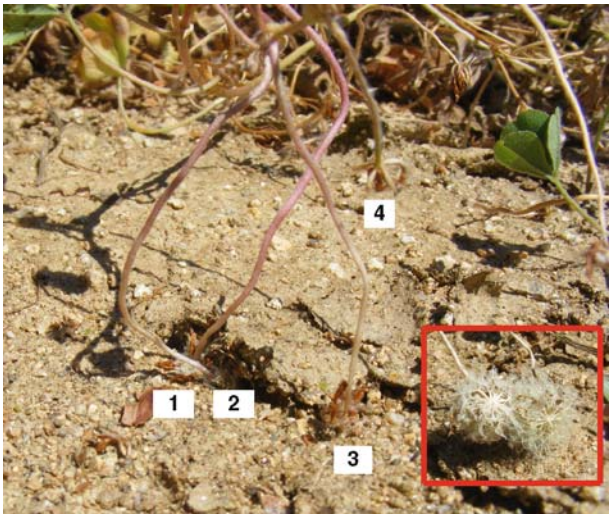

Fig. 1 Burial of reproductive structures in subterranean clover (*T. subterraneum* L. subsp. *brachycalycinum*). By lifting the canopy, four inflorescences are shown: #1 and #2 are already buried and forming a 'burr' (inset is a picture of developed burrs), #3, with backwards bent florets, is just in the phase preceding the ground penetration and #4 is still extending its peduncle towards the ground (Photo P. Fraschini, M. Salis, L. Pecetti)

subsp. *subterraneum* is a 'true burier' and soil penetration by the short and strong peduncle is easily achieved when the soil surface is moist and soft, but it can be limited or prevented when the soil hardens. Conversely, the thin and sarmentous peduncle of subsp. *brachycalycinum* is unable to exert a strong pressure on the surface. Therefore, it elongates until it finds a soil crack or a small shelter, enabling the development of the burr even in heavy and hard soils (Morley and Katznelson 1965). Because of these different adaptation mechanisms, virtually only buried burrs can set adequate seed in the subsp. *subterraneum*, whereas a good part of unburied burrs can fully develop seed in the subsp. *brachycalycinum* if sufficiently protected from the light, and the reduction of seed yield caused by the lack of burial is lower in this latter subspecies.

Seed yield. The seed-producing capacity is the main determinant of success of a subterranean clover genotype (Rossiter 1966) and direct selection for seed yield has been an obvious breeding target, which has always been paralleled by selection for appropriate maturity grading and burial ability. Breeding programmes for semi-arid conditions highlighted that successful strains were characterised by the production of a large number of relatively small seeds (Piano and Pecetti 1996), mimicking the features of adapted natural populations (Piano et al. 1996).

Hardseededness. Breakdown of hardseededness is largely determined by summer temperatures, in particular the range of diurnal temperature fluctuations to which the seeds are exposed (Taylor 1981). The ability of genotypes to maintain adequate reserves of hard seeds over summer (defined as 'residual hardseededness')

depends little on their ability to produce a given proportion of hard seeds at maturity, and there is more inter-genotype variation in the level of residual than initial hardseededness (Piano 1986). Residual hardseededness in natural strains is strongly and positively correlated with the degree of drought and heat stress at sites of origin (Piano et al. 1996). This points to a mechanism of adaptation and survival in environments where the risk of season's false breaks is high. Borrowing from the behaviour of natural strains, breeding has pursued high levels of residual hardseededness for cultivars targeted to environments with short growing season and severe summer stress (Nichols et al. 1996). Insufficient hardseededness is recognised as a major drawback of persistence of subterranean clover in rotation with crops in the ley-farming system, as it limits the regeneration at satisfactory density of the pasture phase.

Grazing tolerance. The geotropy and seed burial, the prostrate habit and the indeterminate branching growth contribute altogether to make subterranean clover a suitable species for grazing (Morley and Katznelson 1965). In breeding programmes, attention is being paid to grazing tolerance by including phases of actual grazing conditions in the selection and evaluation process (Nichols 1993; Piano et al. 1997).

Disease tolerance. Some major disease problems have arisen in Australia since the early 1970s and have been subsequently tackled by the breeding. Clover scorch caused by *Kabatiella caulivora* (Kirchn.) Karak. is the most serious foliar disease. Resistant cultivars were selected (Nichols et al. 1996), but the outbreak of new pathogen races compels to continuous breeding efforts. *Phytophthora clandestina* Taylor et al. is recognised as the main cause of root rot, with *Fusarium* sp. and *Rhizoctonia* sp. being secondarily involved (Barbetti and Sivasithamparam 1987). New races of *Ph. clandestina* appear to overcome known sources of resistance. Transgenic genotypes of subterranean clover were generated by *Agrobacterium*-mediated transformation to enable the expression of antifungal proteins in an attempt to confer novel types of resistance to fungal diseases (Aldao et al. 2000) but this had no impact on actual breeding. Diseases are less important for subterranean clover pastures in Mediterranean Europe than in Australia, possibly as the result of an equilibrium reached gradually between clover populations and pathogens.

Pest tolerance. The red-legged earth mite (RLEM) (*Halotydeus destructor* Tuck.) is the most important pest of subterranean clover in Australia. Breeding for resistance to RLEM has been a major goal in the last decade (Nichols et al. 1996) and progresses were made on identification of compounds with feeding deterrent activity present in the clover leaves.

Yield potential and cool-season growth. In the long term, forage yield of subterranean clover pastures greatly depends on reliability of self-regeneration. Dry-matter yield during a multi-year period of evaluation is ordinarily assessed in breeding programmes (Piano and Pecetti 1996). In addition to overall productivity, attention is also paid to cool-season growing ability and yield. This is a crucial requirement for pastures in Mediterranean environments, where optimum temperature for growth corresponds to optimum moisture only for short periods. Biomass accumulation must be enhanced during the cool season, when the

evapotranspiration is low and the rainfall is probable. Great variation is found among genotypes both for cold tolerance and for autumn–winter dry-matter yield (Carroni et al. 1995), enabling the selection of lines with better cool-season yielding ability (Piano and Pecetti 1996). Cold-tolerant cultivars can also give persistent cover crops in vineyards of non-Mediterranean environments (Pecetti et al. 2007).

Estrogen content and forage quality. Reproductive disorders in sheep, commonly known as 'clover disease', have been related to ingestion of subterranean clover herbage containing high levels of isoflavones with estrogenic activity. Formononetin, genistein and biochanin A were identified as the main isoflavones. Formononetin is usually found in the lowest concentration, but has the highest biological activity and is mainly responsible for the 'clover disease' (Lindner 1967). Low formononetin concentration has therefore become a primary objective of breeding programmes. Through screening of genetic resources (Piano et al. 1997) or dedicated crossbreeding (Nichols et al. 1996) all modern cultivars have formononetin levels lower than 0.20% of leaf dry weight – a threshold considered to be safe for grazing animals. Genotypes with high concentrations of genistein and biochanin A (naturally found) are generating a certain interest for their potential pharmaceutical exploitation (Tava et al. 2006) and as natural growth regulators in animal diets.

Supply of sulphur-containing amino acids increases the wool growth of grazing sheep. The expression of genes coding for sulphur-rich, rumen stable proteins in leaves of transgenic plants was suggested as a potential tool for improving the nutritional value of subterranean clover (Khan et al. 1996).

3 Sulla (*Hedysarum coronarium* L.)

3.1 Biological and Agronomic Features

Sulla is a short-lived perennial (biennial) legume which originated from the central-western Mediterranean Basin and it is the only cultivated species of its genus. Wild populations are commonly found in southern Italy, Sardinia and Sicily islands, northern Tunisia, north-eastern Algeria and southern Spain. The species was domesticated in southern Italy in the 18th century and it is nowadays mostly grown in Italy (where it is the second-most grown forage legume after lucerne) and Tunisia. Sulla is cultivated in rotation with cereals, particularly durum wheat (*Triticum durum* Desf.). In general, it is adapted from loam to clay soils with neutral to alkaline pH. Sulla is substantially dormant during summer and it is grown rainfed in drought-prone areas of southern Italy. In Tunisia, it is mainly grown in temperate areas and it may be irrigated to extend its late-spring production.

Since the 1960s, the species has had a steady decline of cropping area in southern Europe, probably as the consequence of a parallel decline of extensive livestock systems in the most marginal areas where sulla was widely grown, and a generalised adoption of cereal monoculture. In recent years a turnaround is witnessed,

however, with a renewed interest for sulla owing to its renowned assets, such as the mentioned ability to grow in difficult pedo-climatic conditions, the high forage quality and palatability, the ability to improve the soil fertility by its crop residues and the great plasticity of forage exploitation, which may include – even in combination between them – grazing, hay-making, green feeding and ensiling (Stringi and Amato 1998; Minnee et al. 2002). In addition to its high protein content, the forage of sulla is positively characterised by a moderate content of condensed tannins (proanthocyanidins). These polyphenols are able to combine with proteins and other polymers forming stable complexes and may contribute to reduce the degradation of forage proteins in the rumen and the risk of bloat for grazing livestock, while displaying an anthelmintic function (Min et al. 2003). These features make sulla attractive for organic and low-input systems.

Outside the area of its origin, sulla has been introduced into New Zealand, where it is used for grazing in mixture with grasses, as well as for its soil-protecting action (Watson 1982). Two cultivars were developed in this country over the past 15 years, but their adoption has been hindered by high seed costs. The species is raising an interest in Australia too as a deep-rooted perennial forage species, to reduce the recharge of groundwater tables and tackle the problem of secondary dryland salinity (Dear et al. 2003).

Sulla is an outbreeding species – although self-tripping of flowers and self-fertilisation are not prevented (Negri 1987) – forming easily disarticulating pods with tough teguments.

3.2 Major Breeding Goals and Achievements

For decades, the breeding of sulla has been scant and essentially restricted to Italy. Two milestone cultivars were released over 30 years ago, namely the synthetic variety ‘Grimaldi’ and the mass-selected variety ‘Sparacìa’, which were developed in central Italy and Sicily, respectively, from local landraces. A few other cultivars were released more recently. Italian germplasm was also introduced into cultivation in Tunisia about 40 years ago (Annicchiarico et al. 2008), and has since become an important genetic resource for local breeding (Marghali et al. 2005).

The germplasm of sulla shows a wide range of habit forms, from prostrate to erect, which may be exploited in selecting cultivars suited to diversified utilisations. Types with low, dense crowns, tolerant to browsing and trampling and with good budding ability are required for grazing, whereas priority for mowing should be given to vigour, leafiness and rather late flowering to prevent the decline in stem quality observed after blossom. Good quality is compatible, nonetheless, with high yield levels (Minnee et al. 2004).

Consistent with the diversified ideotypes, the two historical Italian cultivars show distinct morphophysiological features, ‘Sparacìa’ being semi-prostrate and adapted to frequent defoliation, and ‘Grimaldi’ being erect, vigorous and suited to forage harvest. Populations originating from areas characterised by high grazing pressure,

Fig. 2 Variation in growth habit and vigour within Italian germplasm of sulla (*H. coronarium* L.) (Photo P. Annicchiarico, L. Pecetti)

such as Sicily and Sardinia, show remarkable frequency of plants with prostrate or semi-prostrate habit (Annicchiarico and Pecetti, unpublished; Figure 2).

Development of at least one prostrate and one erect cultivar for grazing and mowing exploitation, respectively, is also a primary target of selection in Australia (Lloyd et al. 2003). Earlier-flowering cultivars than those bred elsewhere were developed in this country starting from Mediterranean germplasm, to meet the local environmental conditions (Yates et al. 2006). Other specific objectives of breeding in Australia are the selection of appropriate strains of nitrogen-fixing rhizobium (*Rhizobium sullae* sp. nov.), the improvement of *Rhizoctonia* root rot (*Rhizoctonia solani* Kühn) resistance and the selection of cultivars with high seed yield and easy dehulling process to hold down the seed costs. A good seed production may also enhance the persistence of the sward through the large seedling recruitment from the seeds laying on the ground when unharvested (Lloyd et al. 2003).

A good balance of forage yield between the two years of utilisation is a desirable trait, and great germplasm variation occurs for this trait (Stringi and Amato 1998). The influence of biotic and abiotic factors on the level of plant survival into the second year of growth is a matter of great concern. Improved resistance to powdery mildew (*Erysiphe polygoni* DC) is an important breeding target in southern Europe.

The adaptation of sulla to drought-prone areas is a crucial issue worldwide, owing to the role that the species can play in unfavourable environments and the increasing extent of drought events which may be caused by climate changes. Results by Annicchiarico et al. (2008) suggested the need to breed specifically either for rainfed cropping in semi-arid environments or for subhumid or irrigated environments.

Sulla has good growing ability under mild-winter conditions but it is rather susceptible to low temperatures. Better cold tolerance may be useful in the Mediterranean Basin to extend its area of cultivation further north or at higher elevation. Preliminary results showed that great variation for frost tolerance exists among Italian genetic resources of diversified origin (Annicchiarico and Pecetti, unpublished).

Molecular markers, such as AFLP, have been developed in sulla to explore inter- and intra-population genetic diversity and for possible use in marker-assisted selection for important morphological traits (Marghali et al. 2005).

4 Berseem Clover (*Trifolium alexandrinum* L.)

4.1 Biological and Agronomic Features

Berseem, or Egyptian, clover is an annual species grown worldwide, mostly in the Mediterranean Basin, the Indian subcontinent and the southern USA, but also introduced in Australia and South Africa. It is a very important forage legume in warm environments with mild winters, and specifically in countries such as Egypt, Turkey, India, Pakistan, southern Italy and Tunisia. It is generally sown in early autumn and establishes very quickly, thus providing forage production during the cooler months, although the peak of growth is in early spring. It is also grown in colder environments of central Europe, where it is sown in spring. In these environments, it is also used as a cover crop to establish perennial grasslands.

Berseem clover tolerates a wide range of soils, although with a preference for well-drained heavy loams or clays. Some salt tolerance was reported in the species.

Because of its high growing point, berseem clover is not well suited to grazing but it recovers well after mowing, particularly those types characterised by marked regrowth ability as described below. It is considered a very interesting crop for its high forage yield distributed over several mowings (in the regrowing types), good quality (its crude protein content being comparable to that of lucerne), high digestibility and palatability, as well as its positive effect on the soil fertility in rotation with other crops, particularly cereals and cotton. In countries such as India or Tunisia, the winter availability of green feed provided by berseem clover is a basic element for the sustainability of the local dairy industry (Malaviya et al. n.d.). In southern Italy, this crop has the important function to supplement the often poor forage yield of natural pasturelands on which the widespread extensive livestock systems rely.

The species is normally cross-pollinated by insects but considerable variation for self-pollination may occur, as the self-incompatibility mechanism is incomplete and variable among genotypes. Contrasting results were reported for its mating system, ranging from high self-incompatibility to self-fertility (Tasei 1984; Dixit et al. 1989). Tripping of flowers is nonetheless required for pollination and seed set even in self-compatible lines (Roy et al. 2005).

4.2 Origin and Systematics

Berseem clover is unknown in the wild. Its origin and ancestry were examined by Badr et al. (2008) using AFLP markers. They concluded that *T. salmoneum* Mout.

is the likely progenitor species, which evolved into *T. alexandrinum* through artificial selection during the domestication process in Syria, with the possible contribution of *T. berytheum* Boiss. in the same process. After domestication, the early forms of berseem clover may have been taken into rainfed cultivation in Palestine and later into irrigated cropping in Egypt. The Syro-Egyptian germplasm pool is still genetically well differentiated from that of other provenances (such as southern Asia, southern Europe and North Africa), where the species was introduced more recently and likely underwent separate evolutionary processes (Martiniello et al. 1992; Badr et al. 2008).

Two main botanical varieties are recognised in *T. alexandrinum*, namely var. *alexandrinum* Boiss. and var. *serotinum* Zoh. et Lern. (Zohary and Eller 1984), known in the Near East with the local names of 'Fahl' (or 'Fahli') and 'Miskawi' (or 'Miskavi'), respectively. The former has apical branching only and produces one harvest per growing season, whereas the latter, which exhibits basal branching, has an excellent regrowing ability and produces up to six harvests per growing season, provided adequate warmth in winter and moisture in spring are present. Two other, less common botanical forms occur in the species, which are known as 'Saidi' and 'Kadrawi', generally providing two to three harvests per season. The 'Miskawi' form has only gained importance as a crop in India and Italy, because of its outstanding regrowth and its ability to fulfil the needs of the local livestock systems (Martiniello et al. 1992; Malaviya et al. n.d.).

4.3 Major Breeding Goals and Achievements

The first reported case of 'scientific' breeding applied to berseem clover was the one carried out by Jannelli (1972) at the end of the 1960s in southern Italy. The main aim of that programme was a direct improvement of the tolerance to cold and to powdery mildew and an indirect improvement of drought tolerance by selection for early flowering. Through cycles of recurrent selection of local germplasm, the cultivar 'Sacromonte' was selected – featuring enhanced forage yield and stress tolerance – which represented a benchmark of the species for decades. Cold tolerance was seen as a major selection goal in the USA too, to extend the area of cultivation of berseem clover from the original southern states into cooler environments. Through reselection from 'Sacromonte', the cultivar 'BigBee' was eventually released in the 1980s (Knight 1985), with outstanding winter hardiness (surviving winter temperatures of about −15°C) which contributed to the adoption of the species up to the Mid West. Other cultivars were since released in the USA, aiming at more winter hardiness and better yield than in 'BigBee'.

More recent cultivars were also released in Italy, usually after mass selection or recurrent selection from local germplasm grown in coastal central or southern areas. Current breeding programmes in the country are concerned with the improvement of forage and seed yield. As the species is mainly used under multi-cut regimes, regrowth ability is a very important trait for improving forage yield. Selection for dry-matter yield appeared to be more effective in short-cycle (cutting when stems

have seven to eight internodes) than in long-cycle (cutting at 5–10% flowering) harvests (Martiniello and Iannucci 1998). This is a positive finding, as the more frequent harvest rhythm appeared to reconcile good forage yield with optimal nutritive value (De Santis et al. 2004).

In major growing areas such as North Africa and India, berseem clover is usually grown under frequent irrigation. However, in southern Italy and other regions it is grown rainfed. In Australia a minimum of 550 mm rainfall is suggested for high forage production when grown rainfed in southern New South Wales (Hackney et al. 2007). Although its annual cycle should prevent the species from being exposed to severe drought stress, enhanced drought tolerance in spring may be an important requirement for rainfed cultivation. Physiological studies were carried out to support selection of more drought-tolerant germplasm, but the genetic diversity for putatively useful traits seemed to be limited (Iannucci et al. 2000).

Breeding for disease resistance is an important objective in India and Australia (Malaviya et al. n.d.; Hackney et al. 2007). In the former country, the main constraints are root rot (*solani*) and stem rot (*Sclerotinia trifoliorum* Erikss.); in the latter, attention is paid to root rot and clover scorch (*K. caulivora*).

Application of modern biotechnologies is not yet reported in the breeding of berseem clover. However, regeneration of transgenic plants was obtained from hairy roots induced by infection with an *Agrobacterium rhizogenes* strain (Moriuchi et al. 2004).

5 Birdsfoot Trefoil (*Lotus corniculatus* L.)

5.1 Biological and Agronomic Features

Birdsfoot trefoil is a moderately long-lived perennial forage legume native to Eurasia but widely introduced to the rest of the world, particularly in North and South America (USA, Canada, Uruguay). In England its value as a forage crop has been recognised for more than 200 years (Seaney and Henson 1970). In Italy – where the species is ubiquitous in the wild from the sea level to alpine high elevations – cultivation of birdsfoot trefoil was widespread in inner, hilly areas of the peninsula, but it has considerably decreased in recent decades following the abandonment of marginal areas where extensive livestock systems were carried out. Difficulty of harvesting satisfactory quantities of seed also hindered greater success of the species.

Birdsfoot trefoil produces high-quality forage, is moderately tolerant to grazing and does not cause bloating. Although it is generally used in pastures (in mixtures with grasses or other legumes), it can also be used efficiently for hay and silage. It is noteworthy that close grazing removing all stem growth below about 10-cm height can be detrimental to its regrowth and persistence (Bush 2002; Pecetti et al. 2009).

Birdsfoot trefoil is adapted to most soil types, including the poorly drained ones, and tolerates both acidic and alkaline pH. Its better tolerance to acidic soils and

waterlogging than lucerne makes birdsfoot trefoil a potentially useful species for vast areas in Australia (Real et al. 2005). It generally prefers environments with mild summers and annual rainfall of about 500 mm or more (Bush 2002), and it is less drought tolerant than lucerne (Peterson et al. 1992). However, it can be a useful component of pastures in dryland farming systems (Veronesi et al. 1983).

Birdsfoot trefoil (*L. corniculatus* subsp. *corniculatus*) is an autotetraploid species with $2n = 4x = 24$ somatic chromosomes, but some diploid subspecies have also been identified, e.g. *L. corniculatus* subsp. *alpinus*. It is suggested that tetraploid *L. corniculatus* arose from diploid forms through unreduced gametes. The species generally shows an outcrossing mating system by insect pollination (Figure 3); it was reported as self-incompatible by Tasei (1984) and as self-sterile by Richards (1991). However, Veronesi et al. (1983) concluded, also based on previous results, that the genetic system of self-incompatibility may fail in preventing self-fertilisation, thereby resulting in varying levels of outbreeding and inbreeding among and within populations. The lack of efficiency that the incompatibility system sometimes shows may be due to the complex tetrasomic inheritance of incompatibility alleles (Negri et al. 1989). Even in the case of self-fertile genotypes, flower tripping by foraging insects is necessary to break the stigmatic membrane and favour pollination.

Fig. 3 Birdsfoot trefoil (*L. corniculatus* L.) blossom visited by bumble bees (*Bombus* sp.) while large pods have already developed on the same plant (Photo L. Pecetti)

5.2 Major Breeding Goals and Achievements

Two types of cultivars are generally recognised in North America, commonly referred to as 'Europe' and 'Empire', respectively (Seaney and Henson 1970; Bush 2002). The former has faster seedling growth, thicker stems, more upright habit, more determinate flowering period and faster regrowth than the latter (Frame n.d.).

The 'Empire' type includes low-growing, pasture-type cultivars which are most extensively used in North America.

By crossing with wild Moroccan accessions showing the trait, the rhizomatous habit was introduced into cultivated germplasm and the rhizomatous cultivar 'ARS-2620' (or 'Steadfast') was released (Beuselinck and Steiner 1996). Rhizomatous cultivars were intended for pastures and open ranges, as this habit would prevent plants from being fully grazed down, thereby improving their persistence. The rhizomatous habit was also supposedly useful in increasing the persistence of swards in two ways: (i) by new plants (vegetatively generated by rhizomes) replacing dead and diseased plants, particularly in humid areas where the species is prone to root- and crown-rotting diseases; and (ii) by recruited seedlings obtained from seeds set during the season on shoots which had escaped grazing. However, it was shown that rhizomes per se do not assure performance or survival with or without grazing pressure (Beuselinck et al. 2005; Pecetti et al. 2009).

The weak seedling emergence and slow establishment are recognised flaws of the species and should receive greater attention by breeding. Inadequate seed production is caused by two negative features, namely (i) the indeterminate flowering with seed set over an extended period in summer (see Figure 3) and (ii) the easy dehiscence of pods at maturity. Although attempts were made to develop non-shattering cultivars, no significant success was attained under field conditions (Seaney and Henson 1970), so that pod shattering and losses of seed could be reduced only by agronomic interventions (Tasei 1984). Direct selection for seed yield appears promising since large genetic variation and high narrow-sense heritability were observed for seed yield components (Kelman and Ayres 2004).

The persistence of birdsfoot trefoil is limited in humid environments by root and crown diseases, including *Fusarium* root rot [*Fusarium oxysporum* Schlecht. (Snyd. et Hans.)]. Breeding for resistance to this pathogen can be effective and has the potential to increase the field persistence of the crop (Altier et al. 2000). 'Dawn' is a four-clone synthetic variety of the 'Empire' type, specifically bred for resistance to root rot (Seaney and Henson 1970).

Birdsfoot trefoil is a long-day species requiring a minimum daylength of 14 hours for flowering and therefore producing seeds at latitudes of about 40–50°. For regions at lower latitudes such as eastern Australia, cultivars with shorter daylength requirements will favour the natural reseeding in pastures and enhance sward persistence (Real et al. 2005).

Birdsfoot trefoil easily regenerates plants in culture and is respondent to *Agrobacterium*-mediated transformation (Akashi et al. 1998), these features making it a suitable species for biotechnology-supported breeding.

Birdsfoot trefoil possesses adequate concentration of condensed tannins (CT) with all the positive implications that the presence of these compounds determines (Min et al. 2003). CT are compounds derived from the reduction of flavonols and ongoing research aims at isolating the genes of this pathway with the ultimate goal of manipulating it and modulating the level of CT in forage legumes. A particular need is to make CT expressed in those species, such as lucerne, where they are completely absent in feed tissues (Turchetti et al. 2001).

6 Sainfoin (*Onobrychis viciifolia* Scop.)

6.1 Biological and Agronomic Features

Sainfoin is an excellent perennial forage legume, which was widely grown in Europe until the second half of the 20th century. In France it represented 24% of total sown grasslands at the end of the 19th century, being the third species for importance after red clover and lucerne (Huyghe 2006). In Italy it was a traditional component (in rotation with cereals) of rainfed farming systems located on calcareous soils along the Apennine range, where it was mainly used for grazing in autumn–winter and for hay in spring. In Spain sainfoin was largely used in cold, semi-arid and calcareous environments above 600 m altitude (Delgado et al. 2005). Sainfoin is still one of the most widely cultivated forage crops in Turkey, particularly in middle and eastern Anatolia (Tufenkci et al. 2006).

From its Eurasian area of origin, the species was introduced to dry environments of southern and western USA, where it has been widely used for hay or grazing (alone or in mixture with grasses). Its high protein content and palatability, non-bloating forage and fair drought tolerance comparable to that of alfalfa (Peel et al. 2004) make it an interesting species for range improvement for livestock or wildlife (Tilley et al. 2008). Sainfoin is one of the most suited forage legumes to alkaline soils as it is a natural calcicole.

In very recent decades, sainfoin is seen as a resource to be re-discovered for sustainable agriculture in Europe, and this is generating a renewed interest on this species, as it is witnessed by the launching of an international research project specifically devoted to it (HealthyHay 2008). One very appealing feature of sainfoin is its anthelmintic effect (Paolini et al. 2005), which also has a pivotal role in the above-mentioned international project. Sainfoin is rich in both condensed tannins (Goplen et al. 1980) and phenolic compounds (Tava 2005), which proved to have anthelmintic properties in this species (Barrau et al. 2005). Anthelmintic potential of sainfoin hay and silage was confirmed in vivo in sheep and was found to be superior to that of other tannin-rich forage plants such as birdsfoot trefoil (Heckendorn et al. 2006).

Sainfoin is an outbreeding species and according to Tasei (1984) it possesses a self-incompatibility system. However, Negri (1987) reported previous results showing that this system was not necessarily strict and the proportion of self-fertilisation could also be high.

6.2 Origin and Systematics

Two main botanical forms are recognised within the species that are commonly termed as one-cut and two-cut types, respectively. The former (*O. viciifolia* Scop. var. *communis* Ahlef.) is long lasting, remaining productive for 7–8 years, even though the maximum productivity is attained around the fourth year of growth.

However, this type is slow establishing and little vigorous, and it usually provides only one flowering cut per year, the regrowth remaining completely vegetative. The latter type (*O. viciifolia* Scop. var. *bifera* Hort.) is faster growing (even in the sowing year), more vigorous and, if sufficient moisture is available, it is able to provide two to three flowering cuts per year. Its longevity, however, does not usually exceed three growth years. On alkaline soils of inner Italy, the two-cut type proved to have good potential as a short-term grazing crop, and there is scope to exploit the local germplasm to maximise the yield potential and the longevity (Pecetti et al. 2009). Similarly, French two-cut-type landraces showed promising features in their country of origin, particularly in terms of winter growing ability and cold tolerance (Prosperi et al. 1994). Local genetic resources are also being investigated in Spain (Delgado et al. 2008).

6.3 Major Breeding Goals and Achievements

Breeding programmes in North America started in the 1960s, aiming at selecting increased disease resistance, improved nitrogen fixation and higher dryland (single-cut) and irrigated (multi-cut) yield (Tilley et al. 2008). Milestone cultivars such as 'Eski', 'Remont' and 'Renumex' were developed in the 1960s and 1970s in the USA and 'Melrose' and 'Nova' in the 1970s in Canada. 'Shoshone' was recently released for high tolerance to northern root-knot nematodes. Some breeding of the species was carried out in the last decade in southern Italy (Martiniello 2005).

Availability of *in vitro* micropropagation methods (Celiktas et al. 2006) and a microprojectile bombardment protocol (Önde et al. 2001) are recent applications of biotechnological tools, which also offer opportunities for the possible development of transgenic sainfoin plants.

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